CHARLES UNIVERSITY IN PRAGUE
FACULTY OF SCIENCE

BIOLOGY
Terrestrial Ecology

MASTER THESIS

The Influence of Toll-like Receptor 4 Polymorphism on Condition and Ornamentation in Great Tit

Vliv polymorfismu Toll-like receptoru na kondici a ornamentaci u sýkory koňadry

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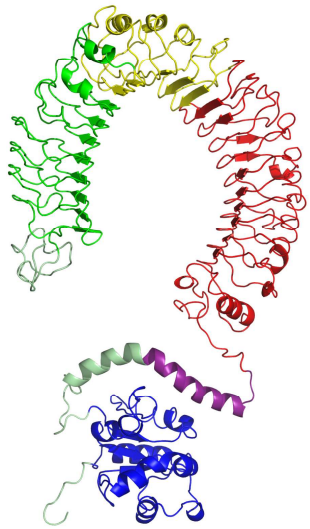
Prague 2011

I. ABSTRACT

Host-parasite co-evolution belongs to the most important evolutionary relationships that shape natural and sexual selection. Parasites pose permanent selective pressure on their hosts. Toll-like receptors (TLRs) as a part of innate immunity are involved in mechanisms of a first immunological barrier which has to be overcome by parasites. These receptors play a key role in primary detection of pathogen-associated molecular patterns (PAMPs) and, hence, are responsible for early triggering of effector immunological mechanisms and for co-activating adaptive immunity. Several studies revealed that TLR4 may represent a suitable model molecule for host-parasite co-evolution studies. TLR4 interacts directly with several PAMPs and structural variability in this receptor was shown to affect host resistance to various diseases. Thus, there is potential for occurrence of parasite-mediated natural and sexual selection. Contrary to the number of fish and mammalian TLRs described, avian inter- and intraspecific TLR variability is only very insufficiently explored. This is especially true for passerine birds. In my diploma thesis I therefore provide the first description of the complete *Tlr4* translated region in a non-model free-living bird, great tit (*Parus major*), predict structure of the protein product of this gene and analyse its population polymorphism. To assess evolutionary forces acting on the great tit TLR4 I described the TLR4 also in several other passerine species. These data were used to investigate influence of the TLR4 polymorphism on condition-related traits in great tit. I found that that one particular amino acid substitution, Q549R, is associated with expression of plumage ornamentation. In both sexes individuals bearing this substitution express narrower melanin-pigmented black breast stripes and lighter carotenoid-pigmented yellow breast colouration. To my knowledge, this is the first evidence for possible association between TLR polymorphism and ornamental colouration in animals. In general, our data show influence of innate immunity on ornamental signalling and support indicator model of sexual selection.



Schematic structure of Toll-like receptor 4.

II. ABSTRAKT

Koevoluce mezi hostiteli a jejich parazity patří k nejdůležitějším ekologickým vztahům, které směřují přírodní i pohlavní výběr. Na hostitele je vyvíjen soustavný selektivní tlak ze strany parazitů a naopak paraziti jsou selektováni kontaktem s imunitním systémem hostitele. První vlnu imunitní obrany hostitele tvoří vrozená imunita, která mimo jiné zahrnuje i Toll-like receptory (TLRs). Tyto receptory jsou klíčové při prvotním rozpoznání molekul asociovaných s patogeny (PAMPs) a jsou tak zodpovědné za spuštění vrozené imunitní reakce a koaktivaci adaptivní odpovědi. Jak ukazují některé studie, TLR4 by mohl být vhodným modelovým receptorem pro studium koevoluce hostitele a parazita. TLR4 totiž přímo reaguje s některými důležitými PAMPs a bylo ukázáno, že strukturní variabilita tohoto receptoru může ovlivnit rezistenci k určitým nemocem. Na TLR4 by tedy mohl působit parazity zprostředkovaný přírodní či pohlavní výběr. Na rozdíl od velkého množství popsaných TLRs u ryb a savců, u ptáků je mezidruhová i vnitrodruhová variabilita TLRs prozkoumána jen velmi nedostatečně, což platí především u pěvců. V této diplomové práci proto poskytuji první kompletní popis translatovaného úseku *Tlr4* u nemodelového volně žijícího pěvce, sýkory koňadry (*Parus major*), dále pak predikovanou strukturu proteinu a analýzu populačního polymorfismu. Aby bylo možno lépe odhadnout evoluční tlaky působící na TLR4 u sýkory koňadry, popsala jsem také TLR4 u několika dalších druhů pěvců. Tato data pak byla použita ke stanovení vlivu polymorfismu v TLR4 na variabilitu v kondičně závislých znacích u sýkory koňadry. Zjistila jsem, že jedna z aminokyselinových záměn, Q549R, je asociovaná s mírou exprese ornamentu. Tato záměna v receptoru ovlivňuje šířku černého melaninového proužku a jas žlutého karotenoidního ornamentu na hrudi a to nezávisle na pohlaví. Tato diplomová práce je první studií zabývající se vztahem mezi polymorfismem v TLRs a ornamentálním zbarvením u zvířat. Obecně pak naše data ukazují, že vrozená imunita může mít vliv na míru exprese ornamentu, a tím podporují indikátorový model pohlavního výběru.

Great tit
(photo: H. Bainová).

III. PREAMBLE

The present thesis is a part of larger project where I am participating. Finally findings result from a relatively rich data set on which were more people interested. Therefore I decided to use often plural in text if I refer the results of this study. Majority of these data were, however, collected with mine contribution. Moreover, I am fully responsible for all presented laboratory part of this study. Most genetic analyses I performed at the Department of Population Biology, Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic in Studenec with the agreement of its head doc. Mgr. et Mgr. Josef Bryja, Ph.D. and under the expert supervising of Mgr. Anna Bryjová a Mgr. Marta Promerová. Several of gene and protein analyses I consulted with my supervisor, Michal Vinkler, who also provided the total lymphocyte count and carotenoid-based ornamentation data. The phenetic tree present in my thesis was generated by Josef Bryja. My co-supervisor, Tomáš Albrecht, helped me with the statistical background of this study. The findings present in my master thesis would be reached only hardly possible without such inspiriting and functional cooperation not only among people but also among scientific institutions.

I declare that the master thesis "The Influence of Toll-like Receptor 4 Polymorphism on Condition and Ornamentation in Great Tit" I wrote all by myself on the basis of the material which is cited in the text and with my supervisor's and colleagues' consultations. This theses or its part was submitted to obtain neither another nor same academic degree.

Prague, 28th August 2011 Hana Bainová

IV. CONTENTS

V. GENERAL INTRODUCTION

Virtually all living organisms are under permanent selective pressure of potentially pathogenic parasites. According to the ecological definition (which is adopted thorough the text of this thesis) parasite is organism that obtain nutrients and some other diverse biological resources from other organisms (hosts) and thus reduces fitness of these hosts (Lawrence 2008). Thus, as parasites are considered various organisms from bacteria to metazoans. Pathogen is then a kind of parasite which is able to cause disease in the host (Clayton & Moore 1997). Any disease may potentially severely decrease an individual fitness due to decrease of their condition and reproductive success (Moller 1997). Therefore, hosts tend to protect their bodies against parasite incursions. Immune system is an important complex of mechanisms protecting host organism against their potential parasites (see, e.g. Danilova, 2006) and thus reducing their negative impact on host fitness. Immune defence, nevertheless, poses substantial costs to the individual. The formation of the immunological barriers as well as induction of various immunological mechanisms recruits resource and energy stores that, hence, cannot be utilised elsewhere, e.g. in growth or reproduction (Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000). It was proposed that this trade-off may mediate a linkage between health, condition and ornamentation (Lozano 1994). Highly ornamented individuals that are in good condition and health state may be those possessing some genetic advantage decreasing their susceptibility to parasites (Hamilton & Zuk 1982). Considerable part of variability in anti-parasite resistance is highly hereditable, determined by immunity-related genes (IRGs; Wakelin & Apanius 1997; Trowsdale & Parham 2004). This heritable nature of the host immunity is important especially from evolutionary perspective as it enables host-parasite co-evolution in time (Woolhouse et al. 2002). Given parasitic selective forces tend to increase host resistance to those particular parasites. This trend is, however, accompanied by micro-evolutionary changes in parasites enabling their survival in the host population. Thus, in evolution occurs a continuous arms race between hosts and their parasites, or in other words „it takes all the running you can do, to keep in the same place“ (The Red Queen´s Hypothesis; Van Valen 1973). Parasites struggle to overcome the host immune defence whereas hosts strive to protect themselves against the widest spectrum of attacking parasites.

In hosts, there are many (about thousand) IRGs. IRGs responsible for detection of parasite attack exhibit extreme levels of polymorphism as a result of rapid evolution due to selective pressures from parasites (Trowsdale & Parham 2004). The main attention is focused

to Major Histocompatibility Complex (MHC) in eco-evolutionary studies. MHC belongs to antigen-presenting molecules and play a central role in self/nonself recognition (Wakelin & Apanius 1997). It was found a relationship between polymorphism in MHC and mating preferences (reviewed in Milinski, 2006). However, to understand the genetic-polymorphism share within the variability in anti-parasitic resistance, it is hardly possible to focus only on one gene cluster. For instance, in humans it was found that at least half of the genetic variability responsible for the resistance to various infections is attributable to non-MHC genes (Acevedo-Whitehouse & Cunningham 2006). It seems that most of these genes participate in innate immunity mechanisms or immunomodulation. Despite recent findings of this kind only little attention has been paid so far to innate immunity in ecological and evolutionary studies (Vinkler & Albrecht 2009). Among these IRGs of the innate arm Pattern recognition receptors (PRRs) may play a crucial role. These receptors are responsible for microbial-component binding and subsequent danger-signalling initiation. PRRs thus belong among first immunity barriers protecting the host against parasites. Several protein families belong to PRRs. These comprise for instance Toll-like receptors, Retinoic acid-inducible gene-I like receptors, Nucleotide-binding oligomerization domain like receptors or The C-type lectin-like domain superfamily (Zelensky & Gready 2005; Kawai & Akira 2009). Toll-like receptors represent undoubtedly a group of PRRs which is currently most extensively studied and described both in structure and function.

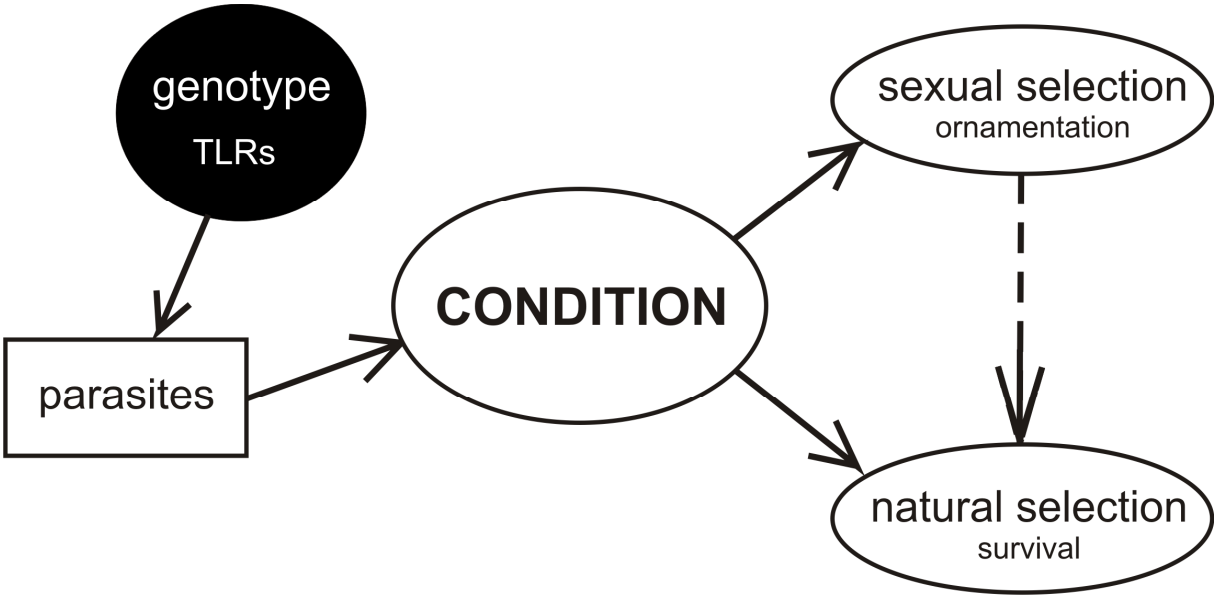


Fig.1 Indirect effect of host genotype (represented for example TLRs) on natural and sexual selection via host condition is mediated by parasite, modified according (Hill 2011).

V.1. Toll-like receptors

The discovery of Toll-like receptors (TLRs) altered much our view on the innate immunity role in anti-parasite protection. It was a research inspiration for many scientists including my supervisor ☺. The first vertebrate TLR was described in 1997 by Medzhitov et al. (1997) as a human homologue of the *Drosophila* Toll. The insect Toll protein is a member of the signalling pathway controlling expression of an antifungal peptide gene which is activated by binding of extracellular host-derived ligand Spatzle on membrane receptor Toll. On the contrary, vertebrate TLRs serve directly for microbe-derived ligand binding (Lemaitre et al. 1996; Medzhitov et al. 1997). TLRs, as innate immunity receptors, are molecules with evolutionally conservative structure and function. They are present in most eumetazoans (Leulier & Lemaitre 2008) and moreover, proteins structurally related to TLRs were recently discovered in unicellular organism as well (Chen et al. 2007). Each vertebrate species is typically equipped with a dozen TLR family members (Roach et al. 2005). The avian TLR repertoire consists of ten genes. TLR2a, 2b, 3, 4, 5 and 7 are common for both birds and mammals, while TLR1a and TLR1b arose by duplication following the divergence with mammalian TLR1/6/10 gene complex. TLR21 seems to be an orthologue of TLR21 found in fish and amphibians and TLR15 is a novel and unique avian receptor (Brownlie & Allan 2011). Individual TLRs differ in their structure and signalling pathways. Each receptor from the TLR family is able to recognize a different and specific set of non-self microbial structures termed 'pathogen-associated molecular patterns' (PAMPs; Kawai & Akira 2009). TLRs are thus responsible for early initiation of an immune reaction, which is essential for successful clearance of potential parasite infections. Immediately after pathogen recognition, TLRs activate specific signalling pathways that lead to inflammation and cytokine production. Eventually this TLR-based immunity regulation co-activates even adaptive immunity through altered expression of various co-stimulatory molecules (Medzhitov & Janeway 2000a; Akira et al. 2001; Uematsu & Akira 2006). TLRs therefore represent one of the functional bridges between innate and adaptive immunity.

Most TLR studies focus mammalian orthologues in various species. In birds the most well known model is the domestic chicken. There are so far just a handful studies that concerned any of the TLRs in other bird species. These comprise of TLR1 in the Griffon vulture, *Gyps fulvus* (de la Lastra & de la Fuente 2007) or with TLR7 in the Pekin ducks,

Anas platyrhynchos (MacDonald et al. 2008). In passerines the TLR4 structure has been reliably described only in Zebra finch, *Taeniopygia guttata* (Vinkler et al. 2009).

V.1.1.Toll-like receptor 4 (TLR4)

Up to now the X-ray crystalographic structure of TLR4 has been described only in humans and mice (Park et al. 2009; Kim et al. 2007). According to these studies the TLR4 molecule belongs to trans-membrane receptors. The extracellular part has a horseshoe-like shape in which the concave surface of the structure is strengthen by β-sheets, and the convex part consists of parallel loops and short helices (Kim et al. 2007). The extracellular part is divided into three subdomains: N-terminal (26-201 aa), central (202-346 aa), and C-terminal (347-629 aa; Kim et al. 2007; Kang & Lee 2011). It consists of leucine-rich repeat (LRR) domains that mediate the binding and recognition of lipopolysaccharides (LPS) from Gram-negative bacteria cells and other ligands e.g. several heat shock proteins (Raetz & Whitfield 2002; Cohen-Sfady et al. 2005; Gay & Gangloff 2007; Kawai & Akira 2009). The process of successful binding and recognition of LPS is quite complex. First lipopolysaccharide-binding protein (LBP) binds LPS released from bacterial cells. This soluble complex of molecules is then brought to host cell membrane where it is recognized by CD14 (Cluster of differentiation 14). CD14 subsequently transfers the LPS molecule to the TLR4-MD-2 heterodimer. LPS is inserted into a pocket formed by the MD-2 molecule. Two LPS-MD-2 complexes are attached from the outer site to the TLR4 dimmer stabilising by LPS interactions the whole LPS-MD-2-TLR4 structure (Park et al. 2009). The LPS binding followed by TLR4 dimerization activates a signalling pathway leading to NF-KB through intracellular interaction of the Toll/IL-1 receptor (TIR) domain in TLR4 with cytoplasmic MyD88 molecule (Akira & Takeda 2004). The intracellular TIR domain of TLR4 seems to be more conserved then extracellular part of TLR4 (Smirnova et al. 2000; Vinkler et al. 2009). TLR4 is expressed in almost all tissues in Zebra finch the highest expression rates were found but most in bone marrow and spleen (Vinkler et al. 2009).

V.1.2.Polymorphism in TLR4

Despite its relatively conserved general structure and function in phylogeny, TLR4 seems to be remarkably polymorphic on the interspecific level (Vinkler & Albrecht 2009). Given its pivotal role in immune protection there is no surprise that TLR4 has been investigated as a candidate gene linked to susceptibility to various diseases. Indeed, the relationship between TLR4 polymorphism and diseases prevalence was demonstrated in many

studies (Misch & Hawn 2008). It is, for instance, known in humans that the presence of a specific TLR4 allele causes high susceptibility to atherosclerosis (Kiechl et al. 2002), endotoxin-associated asthma (Arbour et al. 2000), prostate cancer (Chen et al. 2005) or malaria (Ferwerda et al. 2007). Also in laboratory and domestic animals the TLR4 variability was linked to resistance to various maladies, such as *Mycobacterium tuberculosis* or Parkinson's-like disease in mice (Abel et al. 2002; Panaro et al. 2008), *Mycobacterium avium paratuberculosis* causing Johne's disease in cattle (Mucha et al. 2009) or bacterial infections of the mammary glands in sheep (Swiderek et al. 2006).

Although much information has been collected to the level of population polymorphism in TLR4 in humans, laboratory and domestic animals (Smirnova et al. 2000; Schroder & Schumann 2005; Ferwerda et al. 2007; Misch & Hawn 2008; Jungi et al. 2011), this knowledge is of only limited value in the ecological and evolutionary research. Human population differs from most natural animal populations especially by the way of its exponential growth which weakens much the effect of natural selection (Wlasiuk et al. 2009). Similarly, domestic and laboratory animals are under permanent artificial selection directed by their breeders who may promote other traits than the anti-parasite resistance. The lack of parasite-mediated selection for optimal disease resistance is compensated by the modern medical and veterinary care. Besides, in most laboratory animals the biomedical research utilises only model inbred lines where the variability in genes is almost completely reduced (Leveque et al. 2003). Much more informative are therefore studies focusing the level of interspecies whole-gene polymorphism in non-model animal species. These are, nevertheless, so far rare. Few examples are represented by the recent studies in primates (Nakajima et al. 2008) and cetaceans (Shishido et al. 2010). Similar studies, however, thus far lack in birds. The few avian studies published up to date focused only on the domestic chicken as a model species (Leveque et al. 2003; Consortium 2004; Kannaki et al. 2010; Temperley et al. 2008). Most recently, Alcaide and Edwards (2011) published a paper aiming to describe interspecific as well as intraspecific variability across TLRs in chosen avian species and assess selective pressures posed on them. The basic problem of this study is, however, that it presents only partial, randomly chosen sequences of the coding parts of genes that are unequal in size and localization. Thus, there may be much bias in their results. Hence, we still miss sufficient information about variability in TLRs in free-living non-model birds.

Currently, we do not know much about the associations between polymorphism in TLR4 and prevalence of diseases in birds. The only information available comes from laboratory chicken research. In their study in six inbred lines Leveque et al. (2003) showed that two amino acid substitutions (Y383H and Q611R) in TLR4 are responsible for chicken susceptibility to *Salmonella*. Similar relationship could be possibly found also to other diseases caused by Gram-negative bacteria. Among avian diseases caused by Gram-negative bacteria belong besides salmonellosis (caused by *Salmonella enterica*; Daoust & Prescott 2007) also, for example, avian cholera (caused by *Pasteurella sp*.; Samuel et al. 2007), tularemia (caused by *Francisella tularensis*; Mörner 2007) and borrelioses (caused by *Borrelia sp*.; Olsen 2007). Unfortunately, not much is presently known about avian diseases occurring in free-living bird populations. This is especially true for passerine birds. Even less is known about the genetic polymorphism in these parasite species, although it is much probable that also here broad variability may be present. Thus, much potentially important information still remains to be collected before we can fully understand the importance of the TLR4 polymorphism in evolution of host anti-parasite resistance.

V.2. Natural and sexual selection for anti-parasite resistance

Much of the variability in host anti-parasite resistance phenotype probably arises from the host genetic variation (Ardia et al. 2011). Hence, parasites exert continual selective pressure on the host IRGs. Every particular parasite, nonetheless, directs the evolution of host IRGs in a specific direction. This is due to a distinct set of immune mechanisms responsible for clearance of every particular parasite type. Thus, a trade-off potentially arises among possible directions of the IRG evolutionary adaptations. For example, T-helper 1 (Th1) response is aimed against intracellular parasites, Th2 against extracellular metazoa and Th17 against extracellular bacteria (Jolles et al. 2008; Bradley & Jackson 2008). Particular PRR responsiveness (including various TLRs) may direct the following immune response in a particular Th-type manner (Hawley & Altizer 2011). Specific adaptations in PRRs may therefore favour either Th1 or Th2 or Th17 responsiveness leaving the host less reactive in the other two remaining modes. Evolutionary adaptations in PRRs might therefore trade-off and it depends on the type of parasites that are most important to the host fitness which type of evolutionary changes will be preferred in the evolution of a certain animal population (Klein & Ohuigin 1994; Ferwerda et al. 2007).

Natural selection favours genotypic variants that are most successful in maximising the host fitness. Typically, these variants are those increasing the host resistance (or tolerance) to specific parasites. However, this does not necessarily mean that the immune response to a parasite needs to be maximal. In most aspects the immune defence seems to be rather optimised than maximised. If, for instance, the immune response is too strong, it may damage the host organism and lead to immunopathology (Graham et al. 2005; Woelfing et al. 2009). This may pose some cost to the immunity evolution in hosts. Especially inflammation (which initiates in particular PRR activation) may generate substantial harm to the host as the non-specific reaction may easy get out of control and damage also healthy tissues (Sorci & Faivre 2009; Hawley & Altizer 2011). Thus inappropriately targeted hyper-reactivity of PRRs (including TLRs) may be more detrimental to the host than to the parasite. Additional costs to the host may arise from the energetic and nutritional resources necessary for mounting an immune response (Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Faivre et al. 2003; Alonso-Alvarez et al. 2004; Kilpimaa et al. 2004; Martin 2005). These costs may present a trade-off into the allocation of resources between the immunity and other physiological functions, mainly growth and reproduction (Ots & Horak 1996; Nordling et al.

1998; Norris & Evans 2000; Hasselquist 2007; Martin et al. 2008; van der Most et al. 2011). The balance in these investments is crucial and may be influenced by additional environmental factors such as availability of resources or presence of specific pathogens (Saino et al. 1998; Long & Nanthakumar 2004; Moreno-Rueda 2010; Cotter et al. 2011). We therefore need to take account both evolutionary and ecological costs and trade-offs linked to immune defence if we wish to understand the evolution of IRGs.

Parasite-mediated natural selection may be constrained by the costs of immune adaptations. Hence, the evolution of novel protective variants of IRGs may be rather slow. What might, nevertheless, undergo relatively rapid changes are allele frequencies in individual IRGs. Parasites may be best adapted in their incursion mechanisms to the most common host genotypes (Spurgin & Richardson 2010). This may render the rare host genotypes an advantage in evading the parasites. Sexual reproduction then provides the host major advantage in the possibility of shuffling the genotypes enabling rapid spread of advantageous alleles without the harm of selective sweep in other genetic traits. Sexual reproduction is in most vertebrates associated with some kind of sexual selection that may grant additional benefits from the sexual reproduction and allow even faster changes in allelic frequencies (Flegr 2005).

In sexual selection, the level of individual choosiness is dependent on the level of investments into the single reproduction event (Trivers 1972). Therefore, generally speaking, the more choosy sex is the more investing sex. Females are in most cases the mate-choosing sex, because eggs are presumably more costly to produce than sperms. Furthermore, some males often achieve far more matings than most females, especially in polygynic species, which also decreases their choosiness. On the other hand, in social monogamy, where common care for offspring is necessary, the variance in reproductive success of both sexes is typically almost equal (with exception extra-pair offspring). Thus, in this case also males may exhibit high degree of choosiness (Amundsen et al. 1997; Mand et al. 2005). For simplicity, females will be considered as the choosy sex in the following text although this may not be always the case. Sexual selection is often based on some conspicuous ornamental traits that serve as a criterion of the male quality. I will therefore herein mention several, in my view most important, hypotheses regarding the origin and evolution of ornamentation in female mate choice.

According to Fisherian *Sexy son runaway model* (Fisher 1930) females choose their sexual mates on the basis of a heritable preference for some particular trait (e.g. ornamentation). If this preference is common in the population it is advantageous for males to exhibit this type of ornamental trait as males possessing the trait will obtain relatively higher fitness gains. Thus, if heritable, the genes for the ornamental trait will spread in the population. Under these circumstances it is convenient also for the females to share the genes for the trait preference as their sons will potentially gain higher fitness benefits. This mechanism might ensure fast fixation of both alleles in the population and if it concerns some quantitative genetic variance it may eventually lead to preference for maximal trait expression in females and exaggeration of the ornamental trait in males. Hence, however, this mechanism based on positive feedback should lead to fixation of selected alleles in the population ensuring that all males express the same preferred phenotype. Females would therefore loose their criterion for selection. What can be observed in nature is, nonetheless, the maintenance of variability in ornamental traits. This phenomenon is called *Lek paradox* (Hamilton et al. 2006).

Contrary to the runaway model, the *Handicap principle* (Zahavi 1975) supposes that sexual selection is effective only by selecting a character which lowers the survival of the individual. This character, for example size or conspicuous of ornament, so causes some handicap for their owner, it must be costly produced or costly maintained and it may be consider as a honest mark of male quality. In other words, if the male is able to survive with such handicap, it must be really in good condition. Another and more universal explanation of persist phenotypic male traits variation may give the *Viability indicator mechanism* (Andersson 1994) supposes that the sexual selected trait must be honestly indicator of male quality, with the respect to males viability and health. Females choose sexual mates according to these traits because they hope to obtain some benefits (Hill 1991). As direct benefit for female may be information that males with conspicuous ornament are evidently healthy and the direct risk of infection is lower (*Contagion indicator hypothesis*; Able 1996). Equally important is that these males are in a good condition and thus able to provide adequate paternal care (*Good parent hypothesis*; Hoelzer 1989), i.e. better nest protection or food provision for offspring (Senar et al. 2002; Quesada & Senar 2007). Moreover, it may be assumed that conspicuously ornamented males probably carry some genetic advantage ensuring their health. This genetic advantage may be presence of advantageous alleles of IRGs offering the individual better protection against potential parasites. This genetic material

may provide indirect benefits for female due to her offspring quality (*Good genes hypothesis*; Neff & Pitcher 2005). According to *Parasite-mediated sexual selection* (Hamilton & Zuk 1982), parasites act as significant selective agents on their hosts. Parasites evince negative impact on host condition, so less resistant individuals are in worst condition and thus they can not afford to exhibit conspicuous ornament, which serve as honest indicator of health for female mate choice (Thompson et al. 1997; Brawner et al. 2000; Hill & Farmer 2005). Quality of male could be in this case understood as the ability to defence against parasites. The never-ending co-evolutionary race between hosts and their parasites thus maintain genetic and phenotypic polymorphism in host populations (Klein & Ohuigin 1994; Goater & Holmes 1997).

Birds represent good model taxon for studying sexual selection. Thanks to their excellent sight many avian species developed in evolution various ornamental secondary sexual traits (Hill & McGraw 2006a). The signalling of different ornaments may have other function; some may refer about individual social status, other about condition or health (Thompson et al. 1997; Gonzalez et al. 1999; Quesada & Senar 2007). Below, I will pay attention only to two most common colorations in birds (Hill & McGraw 2006b), one caused by melanins and the second by carotenoids.

V.2.1.Melanin-based ornament

Ornamentation based on melanin pigment is very widespread within birds. This pigment contains molecules with large and highly conjugated structures which are able to absorb day light and UV so it create dark colours (black by eumelanin and brown by phaeomelanin, in birds feathers coexist both forms; Riley 1997; McGraw 2006b). The amount of melanin is not dependent on nutritional diet, contrary to carotenoids (McGraw et al. 2002). Melanin expression in sexual ornaments is caused and controlled by steroid hormones, especially by testosterone (Folstad & Karter 1992; Evans et al. 2000; Buchanan et al. 2001). In birds is very common that sexual ornament is based on melanin. This ornament varies in size mainly among males and is studying in wild-living birds (Gonzalez et al. 1999; Norris 1993). It is known that birds with bigger size of melanin patch are more dominant and aggressive and thus defence their nest intensively than the other males (Jarvi & Bakken 1984; Quesada & Senar 2007). There is a trade-off between investment to feathers growth and to size of melanin ornament, other words in birds with bigger black patch was the feather growth rate slowly (Hegyi et al. 2007) but see (Senar et al. 2003). Birds with testosterone implant

evince higher basal metabolic rate (Buchanan et al. 2001). The size of melanin-based sexual ornament is positively dependent on amount of testosterone and thus might negatively affect immune function (Folstad & Karter 1992; Moller et al. 1996).

V.2.2.Carotenoid-based ornament

Carotenoid-based ornaments are the most studied coloration in birds and in mate choice. Carotenoids are hydrocarbons soluble in lipids. They must be obtained from nutrient intake, birds are not able to synthesize them de novo and the colour of ornament is depend on this intake (Hill et al. 2002; McGraw 2006a). The colour (hue) of the feathers or other bare parts varies from yellow to red (Inouye et al. 2001). The carotenoid-based ornaments play role in mate choice and in social status signalling (see for example (Hill 1990; Hill 1991)). Male with red ornament is more attractive for females than yellow one (Blount et al. 2003). This variability in colouration was attributed just to limited availability of carotenoids in diet (*Carotenoid-limitation hypothesis*, (Grether et al. 1999)).

Carotenoids act as antioxidants which can potentially reduce toxic effect of free-radicals and so decrease oxidative stress in plasma (von Schantz et al. 1999; Mougeot et al. 2010). Carotenoids thus support immunity system because immune cells are highly sensitive to oxidative stress (Chew & Park 2004). It can therefore arise trade-off between allocation of carotenoids to immunity or to sexual selected ornament (*Carotenoid trade-off hypothesis*; Lozano 1994) and this physiologic trade-off guarantee the honesty signalling by carotenoid-based ornament (*Antioxidant role hypothesis*; Perez-Rodriguez 2009). But this could be seen from the other view, that carotenoid-based colouration may signalize health state of individual, because it has enough carotenoids for expression them in plumages as well as for using them in immunity reactions. This theory support many studies (Dufva & Allander 1995; Saino et al. 2000; Horak et al. 2001; Alonso-Alvarez et al. 2004; Peters et al. 2004) but several others do not find this relation (see Dale, 2000; Fitze et al. 2007).

This indicate that the relationship between carotenoids and immune system is probably more complex (McGraw et al. 2006; Peters 2007; Vinkler & Albrecht 2010), for example due to balance exert of testosterone (*Immunocompetence handicap hypothesis*; Folstad & Karter 1992)). Testosterone, as other steroid hormones, stimulates the expression of male secondary sexual characters, but on the other hand plasma testosterone may act as inhibitor of immune functions. Besides this, testosterone may decrease resistance to oxidative stress and thus mediate trade-off among allocation of carotenoids (*Oxidation handicap hypothesis*; Alonso-

Alvarez et al. 2008). Moreover carotenoids are not the only one agent with antioxidative ability (for example vitamin C and E; Royle et al. 2011) and the colourful carotenoid-based ornament may indicate just availability of other antioxidants in plasma standing for carotenoids in antioxidant role (*Red herring hypothesis*; Hartley & Kennedy 2004). Or the honest signal of ornament must be maintained by mechanism that does not rely only on the antioxidant function of carotenoids. The most probable hypothesis for explanation cost and honesty of carotenoid signals in this time seems to be the *Carotenoid maintenance handicap* (Vinkler & Albrecht 2010). In high oxygen concentration (for example during infection and subsequent oxidative stress, or during mitochondrial respiration) are unstable carotenoids damaged because their long aliphatic chains are easily attackable by free radicals. From the helpful carotenoid become harmful derivates which induce inflammatory immune response (Siems et al. 2002). Presence carotenoids in plasma (or extensively expressed in ornamentation) is therefore quite hazardous. Carotenoid-based plumage coloration in birds then reflects the availability of carotenoids at the time of moulting. Honesty of carotenoid-based trait is then in balance between positive function of carotenoids in reducing free-radicals and damaging effect of carotenoids derivates in plasma.

TLR4 could be a candidate gene for resistance to many of diseases. Variability in such IRG that interact with specific molecular structures of parasites may be responsible for differences in host resistance and thus it could influence the parasite-mediated sexual selection. Resistance or susceptibility to diseases due to TLR4 does not directly cause high mortality, but it may only relatively increases or decreases condition of an individual. It is well demonstrated that activation and maintenance of immune system is costly and this cost can be mirror in decreased reproductive success due to express worse secondary sexual traits, which may be mirror in decreased total fitness (see for example Faivre et al., 2003; Peters et al. 2004; Alonso-Alvarez et al. 2004). If indicator hypothesis is valid, then condition significant TLR4 polymorphism could influence expression of sexual ornament and thus sexual selection, reproduction and total fitness of individual.

VI. AIMS AND HYPOTHESIS

In my diploma thesis I decided to expand the current knowledge to the TLRs structure, polymorphism and function in non-model species of birds. As a model organism I chose passerine birds as these comprise more than half of all avian species. Special attention was paid to great tit as this species possess a colourful ornamentation consisting from both melanin- and carotenoid- pigmented traits. As a target gene I chose TLR4 because the structures of the gene as well as its protein product are well described model species and the detailed immunological function of the receptor is also known. Moreover, polymorphism in this gene was previously shown to modify resistance to bacterial infections in the domestic fowl (Leveque et al. 2003).

Aims:

- Describe the *pmTlr4* gene
- Predict the pmTLR4 receptor structure
- Reveal polymorphism in pmTLR4
- Find signatures of possible selection in the gene
- Map possible linkage between *Tlr4* variability, condition and ornamentation in great tits

Hypotheses:

- Structural variability in TLR4 on an interspecific level does not follow the phylogenetic relationships among species completely.
- Even within species there are sites in pmTLR4 under positive selection.
- These sites are associated with differences in individual condition and ornamentation.

VII. METHODS

VII.1. Model species: great tit (Parus major)

Great tit (*Parus major*) is a small passerine, abundant in woodlands and in city parks in Czech Republic as well as in the whole Europe (Cramp et al. 1994). It is mainly a sedentary species forming in our latitude flocks during winter that roam through the countryside (Cepák et al. 2008). The colouration of great tit is conspicuous and partially dichromatic. Both sexes express melanin-based and carotenoid-based feather ornamentation. However, females differ from males in the lighter yellow breast plumage colouration and narrower central black breast stripe (Norris 1990; Svensson et al. 1999). These ornaments are under sexual selection (Jarvi & Bakken 1984; Dufva & Allander 1995; Senar et al. 2003; Hegyi et al. 2007; Isaksson et al. 2008). Females prefer to mate with males possessing wider melanin-pigmented stripe and yellower carotenoid-pigmented plumage (Norris 1990; Kingma et al. 2008). Both ornamental traits are condition depend (Mand et al. 2005). Norris (1990) has studied a breeding great tit population and reported that males with wider black central stripes paired preferentially with females that laid previously large clutches. Simultaneously, there was, nevertheless, no significant relationship between the male stripe width and quality of his territory. This might indicate that the trait is at least partially independent on male dominance. Cross-fostering experiments manifested that the size of the black stripe is heritable and that there is a positive correlation between stripe size in a putative father and survival of his male offspring (Norris 1993). Thus, the expression of this condition-dependent trait has an genetic background. This has been confirmed also by the results of Senar and Quesada (2006) who found that the size of melanin-based plumage is more heritable trait than the chest carotenoid-based colouration which seems to represent a condition depend trait. The size of the black stripe is age-depend, at least when comparing old birds with first-years (Hegyi et al. 2007). This result could, however, indicate selection against less ornamented individuals. Both types of ornamentation in great tits were found in relationships with individual health (Dufva & Allander 1995; Horak et al. 2001). Therefore great tit seems to be a suitable model species for studying influence of variability in IRGs to condition or ornamentation.

VII.2. Animals

Great tits were caught in 2006-2010 during late winter months (from late January to early March), i.e. in their pre-breeding season. The study site was located in an old orchard at the Prague outskirts (Bohnice; N 50°7´24.779", E 14°25´55.924"; see the Fig.2). In my master thesis I have used data from 50 individuals comprising both males and females of mixed age categories. For inter-specific comparison I have included samples from other six passerine species: great reed warbler (*Acrocephalus arundinaceus*), common rosefinch (*Carpodacus erythrinus*), collored flycatcher (*Ficedula albicollis*), barn swallow (*Hirundo rustica*), bluethroat (*Luscinia svecica*) and house sparrow (*Passer domesticus*). In these species one individual per species was used sampled in 2010 in the Czech Republic.

VII.3. Data collection

All birds were captured into mist nets according the standard protocol given by the Ringing station of the National Museum in Prague. Immediately after capture a sample of blood was collected from wing vein in each great tit individual (ca 100 μl, a minimal amount of blood needed for the following analyses). First, 15 μl of blood were transferred into 2985 μl of Natt-Herrick solution (Campbell & Ellis 2007) and stored at a cold place until its transfer into the laboratory. These samples were later used for absolute leukocyte count assessment (Lucas & Jamroz 1961). Second, the rest of the blood sample was transferred into ethanol and later used for genetic analyses. Then basic condition indicators were recorded. In each individual the body weight was measured using digital balance (Pesola PPS200, accuracy to 0.02 g) and the length of tarsometatarsus (a good predictor of individual body size; see Senar & Pascual, 1997) was measured with digital calliper (Kinex, type 6040.2, accuracy 0.01 mm). The second tail rectrix from the left side was collected for ptilochronological assessment of feather growth bar width (Grubb 2006). In 48 individuals the ventral part was afterwards scanned under standardised conditions with Epson scanner V300 to allow later assessment of the ornament expression. All images were taken with colour, grey and size standards in a dark tent in a 24 bit colour mode. In other species only the blood sample for genetic analyses was collected. Finally, each individual was ringed with an aluminium ring of the Ringing station of the National Museum in Prague with unique numeral combination. This approach prevented repeated sampling of the same individuals.

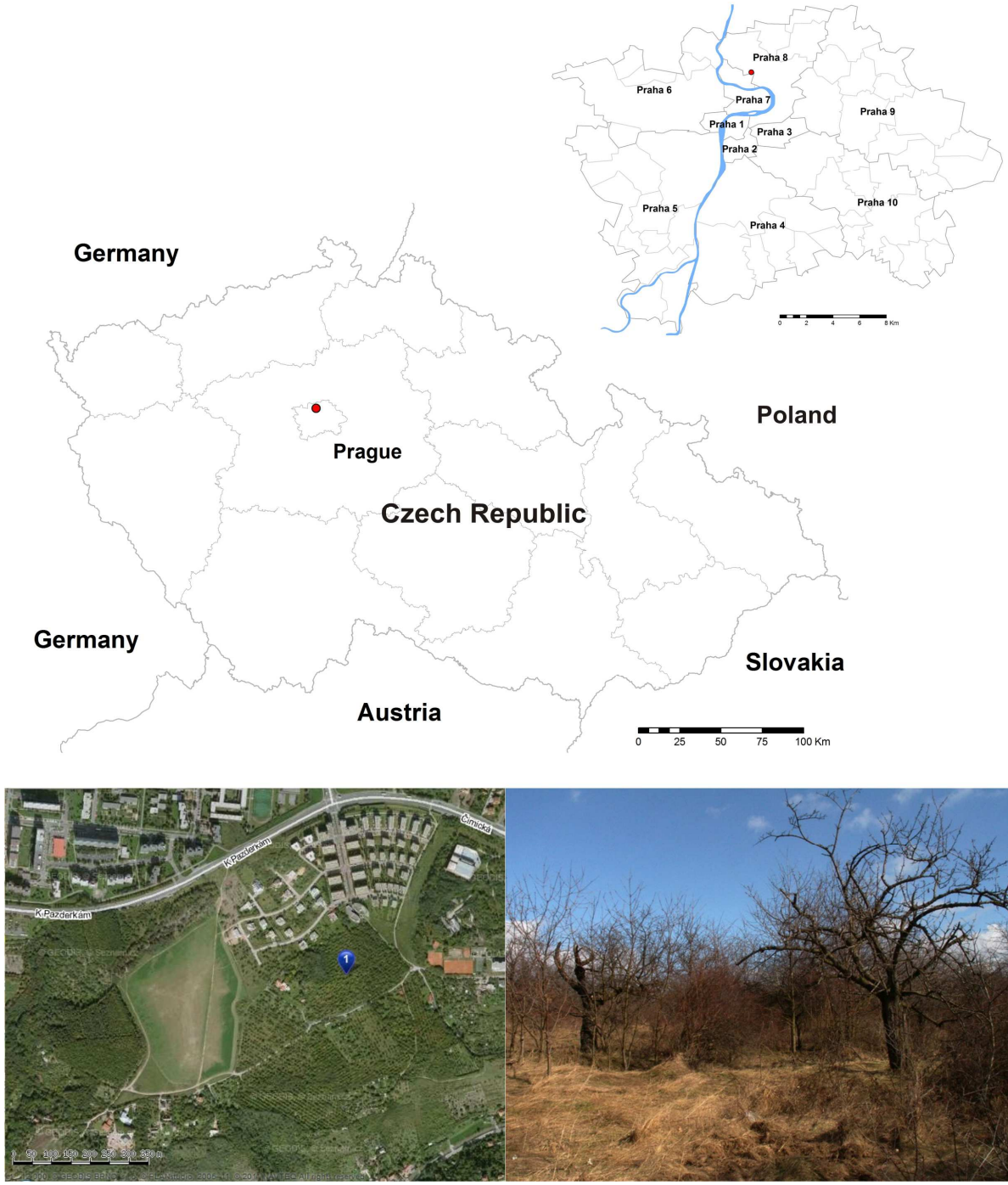


Fig.2 Zoom on **the locality in Prague Bohnice**.

VII.4. Genetic analysis

DNA was extracted from all blood samples with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Primers were designed using Primer3 web tool (http://frodo.wi.mit.edu/primer3/; Rozen & Skaletsky 2000) and on the basis of conservativeness of *Tlr4* gene sequence in zebra finch (tgTLR4; Vinkler et al. 2009) and chicken (ggTLR4; Leveque et al. 2003). The positions of both amplification and sequencing primers are listed in Tab.2 (and shown in S3 in supplementary data) and their specific sequences and polymerase chain reaction (PCR) conditions are given in Tab.1. The whole translated region of *Tlr4* (exon 1, 2 and 3) was covered by two long range PCRs (2828 bp and 2522 bp). During the optimization step two different polymerases were used: Taq DNA Polymerase (Fermentas) and HotStar Taq DNA Polymerase (Multiplex PCR Kit, QIAGEN) with touch-down PCR and heminested PCR modification. Successfulness of PCRs was checked using gel electrophoresis. Amplified PCR products were purified using ExoSAP-IT PCR Clean-up Kit (GE Healthcare Life Sciences) and then amplified with sequencing primers using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Finally, these products were purified with BigDyeXT Terminator kit (Applied Biosystems). Prepared PCR products were then sequenced using ABI 3130 Genetic Analyser (Applied Biosystems). To minimise PCR artefacts the final sequence of *Tlr4* was composed from at least two (ideally tree) independent partial sequences obtained using different sequencing primers. In 10 great tit individuals and all other passerine species cloning was performed to obtain sequences of individual alleles. For cloning CloneJET™ PCR Cloning Kit (Fermentas) and home-made competent *Escherichia coli* cells of strain JM109 (Fermentas) were used. Chosen clone colonies were amplified and PCR products were sequenced using the same protocol as the one mentioned above. In the following text *Tlr4* gene sequences of all included species are abbreviated using the first letters from their scientific name, i.e. *aaTlr4* in *Acrocephalus arundinaceus*, *ceTlr4* in *Carpodacus erythrinus*, *faTlr4* in *Ficedula albicollis*, *hrTlr4* in *Hirundo rustica*, *lsTlr4* in *Luscinia svecica*, *pdTlr4* in *Passer domesticus* and *pmTlr4* in *Parus major*. Proteins are abbreviated analogously in regular capitals (e.g. pmTLR4).

Tab.1 PCR optimal conditions used to amplification of two products which cover whole translated region of *pmTlr4*.

Forward primer	Reverse primer	Product length bp	Temperature ℃	Extension time m:ss	No. of cycles
pmTLR4-1F	pmTLR4-3R	2828	62	2:50	33
pmTLR4-3F	pmTLR4-6R	2522	60	2:40	33

Tab.2 Summary of primers used for PCR (positions of primers are given according to pmTLR4 coding sequence).

Primer name	Primer sequence	Position
pmTLR4-1F	5´- GTCTCCAGGTTTCTCTCTCCATG - 3´	-55
pmTLR4-1R	5´- CCAAACAGGGATCCAGGAGG - 3´	103
pmTLR4-2F	5´- GCACTGGACTGAACATCTCTGG - 3´	131
pmTLR4-2R	5´- CTTGAGAGATCCAGAAGCTGC - 3´	272
pmTLR4-3F	5´- CCTGGGGAAAGCAGCTTTCTATG - 3´	360
pmTLR4-4F	5´- CAGAGGAGACCTTGATGCCCTG - 3´	585
pmTLR4-3R	5´- CCCTCAGGGCATCAAGGTCTCC - 3´	611
pmTLR4-4R	5´- CTGACCTGCAAACCAGCCAGG - 3´	777
pmTLR4-5F	5´- TGTGCCAGGTACAGATGGAA - 3´	848
pmTLR4-6F	5´- CTGCTGCTCCCCTCAGTTT - 3´	1191
pmTLR4-5R	5´- CCAAGGTGTGGAGGTGACTT - 3´	1529
pmTLR4-7F	5´- TGCCCTCTCTGAGCTAAAGGAG - 3´	1575
pmTLR4-8F	5´- CCAACGTGAGCCTGCCAAG - 3´	1871
pmTLR4-6R	5´- GGGTAAATGGTCTCAGGACCC - 3´	2882

VII.5. Protein structure

All obtained sequences were first analysed in Sequencing Analysis, version 5.2 (Applied Biosystems). Subsequently, positive partial sequences were composed into contigs in SeqScape version 2.5 (Applied Biosystems). In BioEdit Sequence Alignment Editor (Tom Hall, Ibis Biosciences; Hall 1999) were then these complete sequences of *Tlr4* translated into TLR4 amino acid sequences. In pmTLR4 all further descriptive analyses were done based on allele 1 of individual 10075. On the basis of the obtained TLR4 sequence domain composition was predicted using SMART web tool, version 5.0 (Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de/; Schultz et al. 1998; Letunic et al. 2009). The signal peptide cleavage sites were predicted in web tool SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/; Bendtsen et al. 2004). The recombination breakpoints were estimated in GARD web tool (www.datamonkey.org; Pond et al. 2006b). The secondary and tertiary structures were predicted using PHYRE2 web tool (Protein

Homology/analogY Recognition Engine version 2.0; http://www.sbg.bio.ic.ac.uk/phyre2/; Kelley & Sternberg 2009). Final visualisation was done in PyMOL, version 1.4.1.

VII.6. Assessing polymorphism

Intraspecific single nucleotide polymorphism (SNP) sites were identified by repeatedly found double peaks in SeqScape version 2.5 in all 50 individuals and also in other investigated passerine species. In 10 great tit individuals and in other passerine species this information was verified also by cloning. Number of SNPs within our population sample was visualised in FaBox web tool, version 1.35 (www.birc.au.dk/software/fabox/). The SNPs were then manually divided into the synonymous and non-synonymous ones. In great tit we reconstructed the sequences of all individual pmTlr4 alleles using PHASE algorithm (Stephens et al. 2001) implemented into the DNAsp software, version 5 (Librado & Rozas 2009) based on the information on allele sequences from the 10 cloned individuals. Non-synonymous substitutions were then grouped into conservative and non-conservative based on the individual amino acid properties. In this study a group of alleles with the same amino acid sequence was termed as a haplotype.

VII.7. Evolution in passerine TLR4

The similarity of pmTLR4 and TLR4 in other investigated passerines to TLR4 in model species (zebra finch, tgTLR4, ACN58232.1; chicken, ggTLR4, ACR26281.1; mus, mmTLR4, AAH29856.1; human, hsTLR4, AAY82267.1) was assessed using the BLAST web tool (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/) and determined as identities (considers identical amino acids in the alignment) and positives (considers similar amino acid in the alignment). The impact of selection on TLR4 was estimated based on comparisons of dN/dS ratios (the number of non-synonymous substitutions per non-synonymous site to the number of synonymous substitutions per synonymous site) in REL analysis (Pond & Frost 2005) at interspecific level and in IFEL analysis (Pond et al. 2006a) at intrapopulation level in great tits using the Datamonkey web tool (www.datamonkey.org; Delport et al. 2010). The avian TLR4 phenetic tree was constructed from the amino acid alignment (all known passerines plus chicken as outgroup) using the neighbour-joining method with options of pairwise deletion, Poisson correction and different evolutionary rates with a gamma parameter of 1 in MEGA software version 4.0 (Molecular Evolutionary Genetic Analysis, http://www.megasoftware.net/; Tamura et al. 2007). The reliability of branching was tested by 1000 bootstraps.

VII.8. Condition dependent traits

Absolute leukocyte count was chosen as a haematological parameter indicating individual health state. In 48 great tit individuals a blood sample of 15 μl was diluted in 2985 μl of Natt-Herrick solution (see Campbell and Ellis, 2007) and left for several hours in a field refrigerator to stain the cells. Thereafter, the numbers of cells were counted in a Bürker's counting chamber (100 large squares were scanned). The leukocyte counting was performed only by one person, Michal Vinkler, to minimise any potential artefact variability among the measurements.

Mean growth bar width was evaluated as a ptilochronological marker indicating individual nutritional condition in the time of moulting. During moult, bird feathers grow in regular daily cycles forming the 'growth bar pattern'. Width of one grow bar thus provides a record of feather grow during a 24 hour period (Grubb 2006). A similar approach was used e.g. by Hill & Montgomerie (1994) in male house finches (*Carpodacus mexicanus*). The left second outermost restrices pulled out from 48 investigated individuals were scanned with a size standard by scanner Benq, type 5550T and in the grey scale reflex mode with 600dpi resolution. Digital images were adjusted in Corel PHOTO-PAINT X3 software by function 'Local Equalization' (with parameters Width 100 and Height 100) which markedly improved the visibility of the growth bars (see Fig.3). The adjusted digital images were used to measureme the total rectrix length and the mean growth bar width in Image Tool software, version 3. To estimate the mean growth bar width a segment of 10 growth bars was measured with the centre in 2/3 of the feather (Grubb 2006) and divided by 10. All restrixes were measured only by myself, to minimise any potential artefact variability among the measurements.

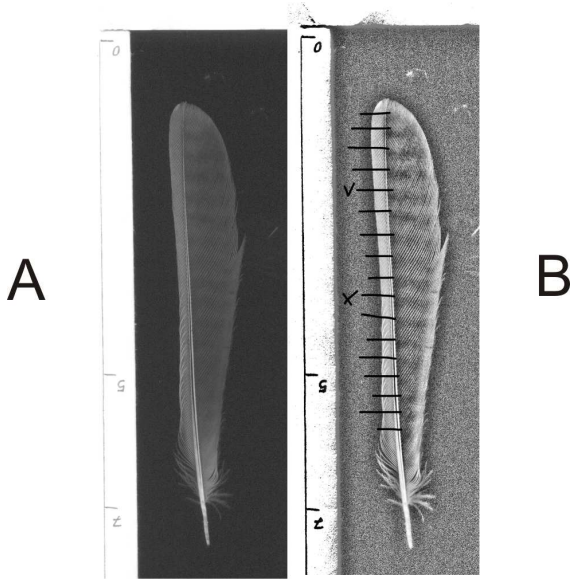


Fig.3 The comparison of **growth bars visibility** before (A) and after (B) modification in Corel PHOTO-PAINT.

VII.9. Ornamentation

The area of the central black melanin-pigmented breast stripe was measured from digital images of each investigated individual (obtained as mentioned above in subchapter VII.3.) in Adobe Photoshop 5.0 (Adobe Systems Inc., San Jose, California). The stripe was measured always only in the length of 5 cm from the neck (see Fig.4). Black pixels of the breast stripe were automatically selected and the area was calculated according to the size standard (in mm^2). All these measurements were performed by myself to minimise any potential artefact variability among the measurements.

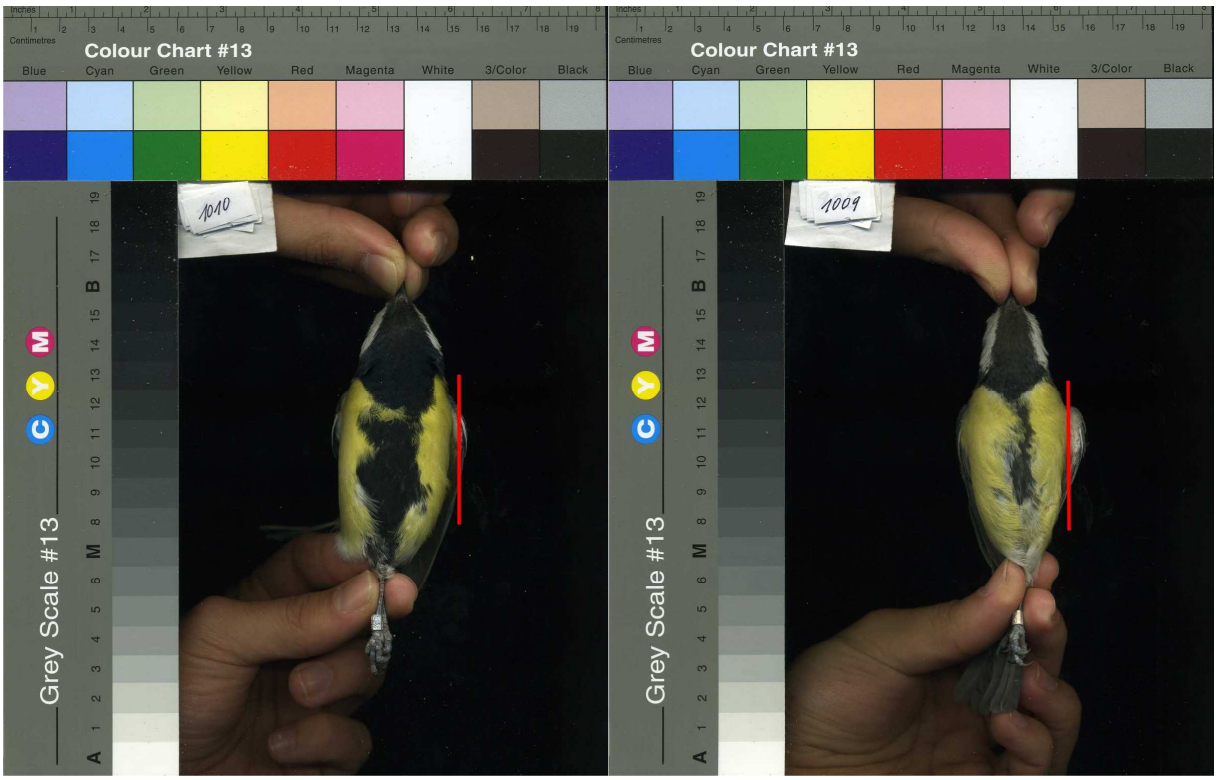


Fig.4 Two **images obtained by scan** the individuals. The red line is 50 mm long and is correspond with length of black measured area.

The yellow carotenoid-pigmented breast colouration was also analysed from digital images. All images were first standardised in their colouration in Adobe Photoshop 5.0 based on the colour standards. Then the average colour of 10 spots (each of the area of 11x11 pixels) evenly distributed in the yellow part of the breast ornamentation was evaluated. The outputs were recorded in HSB (hue, saturation and brightness) colour spaces and average values estimated from the 10 measurements were calculated. Hue (with higher values in more yellowish colouration) is in finches indicative of the type of carotenoids present in the

feathers (Inouye et al. 2001); saturation was shown to reflect the quantity of the carotenoid content (Saks et al. 2003) and lightness appears to be determined by the structure of the feather microsurface with higher values in degraded feathers (Shawkey et al. 2007).

VII.10. Statistics

Statistical analyses were performed using the statistical software R version 2.9.2 (R Development Core Team 2009). To analyze effects of individual frequent non-synonymous non-conservative substitution on hue, saturation and lightness of feather colouration, breast stripe area, body mass, individual size, absolute leukocyte count and mean growth bar width general linear models were used. Sex was treated as a factor variable co-explaining variability in the condition-dependent traits. Prior to analysis, data were checked for normality in continuous variables using Kolmogorov-Smirnov test. Minimum adequate model (MAM; i.e. a model with all terms significant) was obtained by backward eliminations of particular terms from the full model. Candidate models were compared based on the change in deviance with an accompanied change in degrees of freedom using F statistics. The presented significances in MAM are based on Type III Sum of squares. Standard statistical tests were used otherwise. The significance level was set to $P = 0.05$. Comparison of observed frequencies with expected frequencies was performed using the Chi^2 test. Correlations were tested using the Pearson's product-moment correlation.

VII.11. Ethical note

The research was approved by Prague Municipality Department of Environmental Protection (SZn. MHMP-0188194/2010/OOP-V-107/R-37/Pra) and Ethical committee of the Faculty of Science, Charles University in Prague (licence No. 276669/2007-30).

VIII. RESULTS

VIII.1. Description of structure TLR4 in Great tit

The total length of coding sequence of the *pmTlr4* gene is 2526 bp (beginning with initial methionine, ATG and ended by TGA stop codone) which is predicted to translate into an 842 aa receptor protein. The gene consists of three exons (length 105 bp, 167 bp and 2257 bp respectively). Using SMART web tool the pmTLR4 domain structure has been estimated (Fig.5 but see also Fig.6). The predicted receptor molecule consists of a large extracellular domain (637 aa), a transmembrane domain (22 aa) and an intracellular domain (183 aa). In the extracellular domain 9 LRRs (mean length 23 aa) and LRR-CT (50 aa) were predicted. The large part of the intracellular domain is formed by the TIR domain (146 aa). Presence of a signal peptide at the N-terminal part of the extracellular domain was identified by both Neural networks and hidden Markov models with the most likely cleavage site positioned between 27-28 aa and 30-31 aa respectively (mean S score = 0.850, signal peptide probability is 0.995). Two recombination breakpoints in *pmTlr4* were detected (452 bp and 1568 bp, p = 0.01). The predicted pmTLR4 shared maximum similarity with tgTLR4 (almost 90%; see Tab.3). The most probable secondary and tertiary structure was estimated using PHYRE2 web tool.

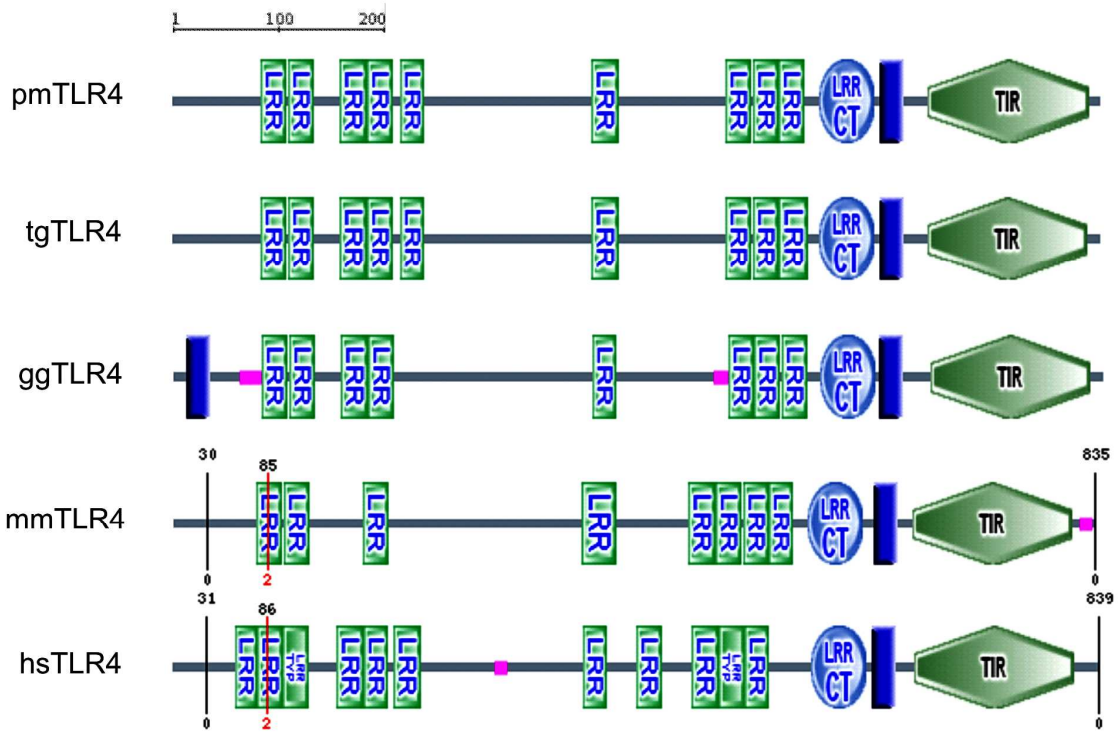


Fig.5 Comparison of **predicted domain structures** in pmTLR4 and selected model species using SMART web tool.

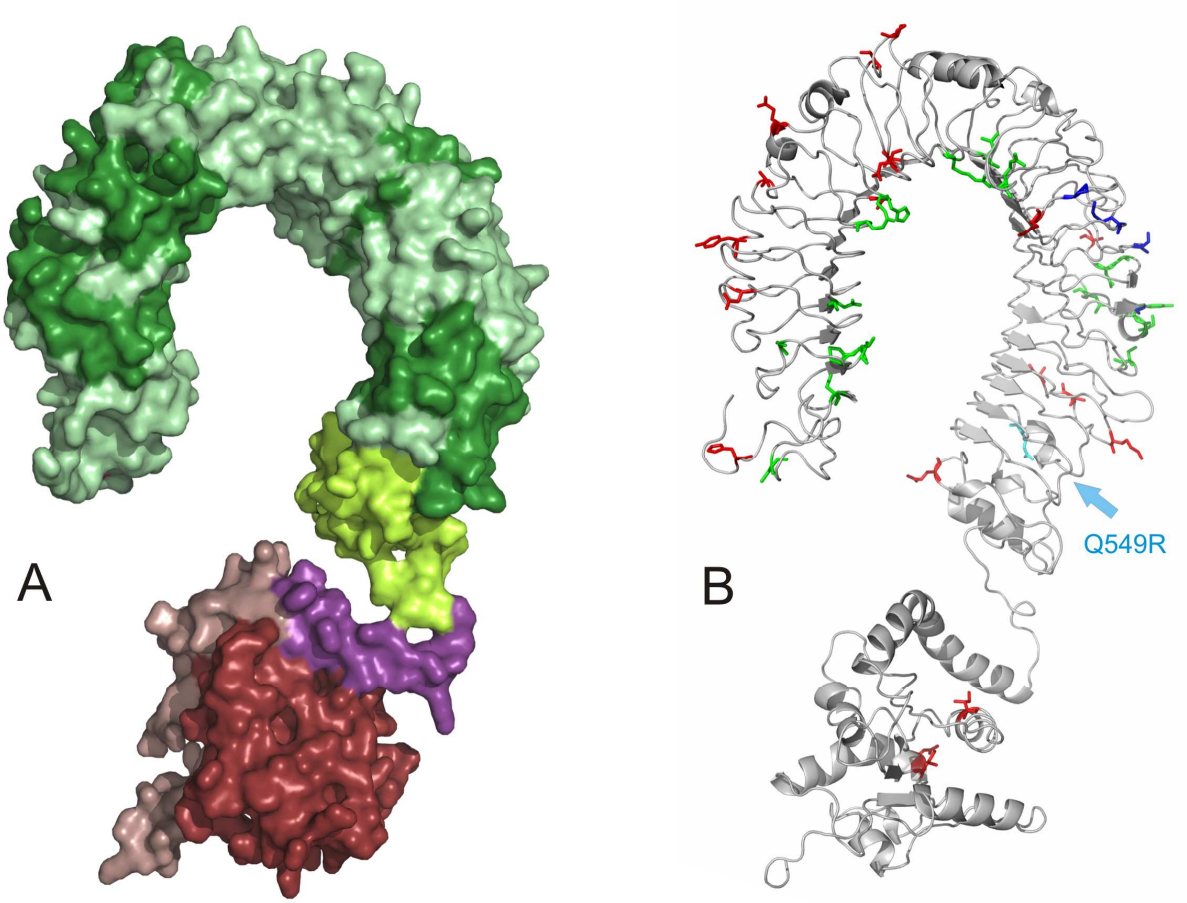
Tab.3 **Predicted identities and positives** on basis of amino acid blast pmTLR4.

	NCBI ID	Identities (%)	Positives (%)
tgTLR4	ACN58232.1	89	93
chTLR4	ACR26281.1	75	85
musTLR4	AAH29856.1	44	62
homoTLR4	AAY82267.1	47	64

VIII.2. Population polymorphism in pmTLR4

A very high rate of variability was revealed in *Tlr4* in Great tit. Each of the 50 individuals sequenced was determined as a heterozygote based on the nucleotide sequence. In total, we found 82 alleles and 54 SNPs were detected. Among these SNPs 34 represented synonymous and 20 non-synonymous substitutions (the list of all SNPs is provided in supplementary data as S1, and amino acid substitutions in S2 and also in Fig.8; 3D model of the protein indicating positions of amino acid substitutions is given in Fig.6). Non-synonymous amino acid substitutions are marginally significant more frequently in LRR motives than in other TLR4 regions ($p = 0.06$). On the basis of amino acid sequences 42 non-synonymous haplotypes were constructed from the identified alleles. Of the 20 amino acid substitutions differentiating the haplotypes 5 were conservative with no significant change in the residue physical or chemical properties. The other 15 substitutions were found non-conservative and, thus, with potential influence of the altered residue on the protein structure and function. Among the haplotypes only three reached the frequency more than 5% (with maximal detected frequency 12% in hap7 and hap12), 7 haplotypes had their frequency between 2,5-5% and the rest of the haplotypes were represented in the Great tit population in the frequency of 2,5% or less.

Fig.6 (see on the following page)
Tertiary pmTLR4 structure prediction. The surface model (A) with illustrated extracellular domain (green), transmembrane part (violet), intracellular domain (red), LRRs (dark green), LRRCT (yellow-green) and TIR (dark red). The model (B) with predicted alpha helix (helixes) and beta sheet (arrows) structure with highlighted polymorphic sites in pmTLR4 (red) and other important amino acid sites: MD-2 (green) and LPS (blue) binding positions. The crucial amino acid substitution is highlighted azure blue and moreover by the arrow (see also Fig.11).



VIII.3. Evolution in pmTLR4

To assess footprints of negative selection the individual haplotypes recognised in the Great tit population were divided based on their frequencies into common (4 or more occurrences) and rare (less than 4 occurrences). We revealed that based on this division the haplotype distribution matched the expectations given by the Hardy-Weinberg equilibrium ($Chi^2 = 2.153$, $p = 0.341$). The haplotypes were then grouped according to the frequencies of their SNPs into common-variant haplotypes (all SNPs found in more than 4 copies in the population) and rare-variant haplotypes (at least one substitution found in less then 4 copies). Although we never detected two rare-variant haplotypes in a heterozygote state in one individual also in this case we found that the results are consistent with the expectations under the Hardy-Weinberg equilibrium ($Chi^2 = 2.250$, $p = 0.324$). Hence, the minor haplotypes and haplotype variants did not exhibit any deleterious effect in homozygote state. Taken per individual sites, IFEL population analysis which is based on non-synonymous/synonymous ratio (dN/dS) revealed in *pmTlr4* 4 positively and 15 negatively selected sites at default

significance level p=0.100. REL interspecies analysis revealed 15 positively and 55 negatively selected sites in passerines *Tlr4* (Fig.7; at the level of Bayes Factor = 20). Interspecific comparison of pmTLR4 to TLRs in model species has revealed high similarity in all sequences. Shared was also the overall predicted secondary and tertiary structure (see supplementary data, S4). The phenetic tree constructed based on the neighbour-joining method has shown that aa sequence similarities are not consistent with the known phylogenetic relationships in passerine birds (Barker et al. 2002; Fig.8). Detailed analyses of evolutionary rates in individual domains in passerines indicated solver substitution rates in MD-2 binding and TIR regions while relatively higher frequencies of substitutions were found in the ligand-binding central domain. The estimated aa similarities of all passerine TLR4 sequences described to consensus tgTLR4 are shown in Tab.4.

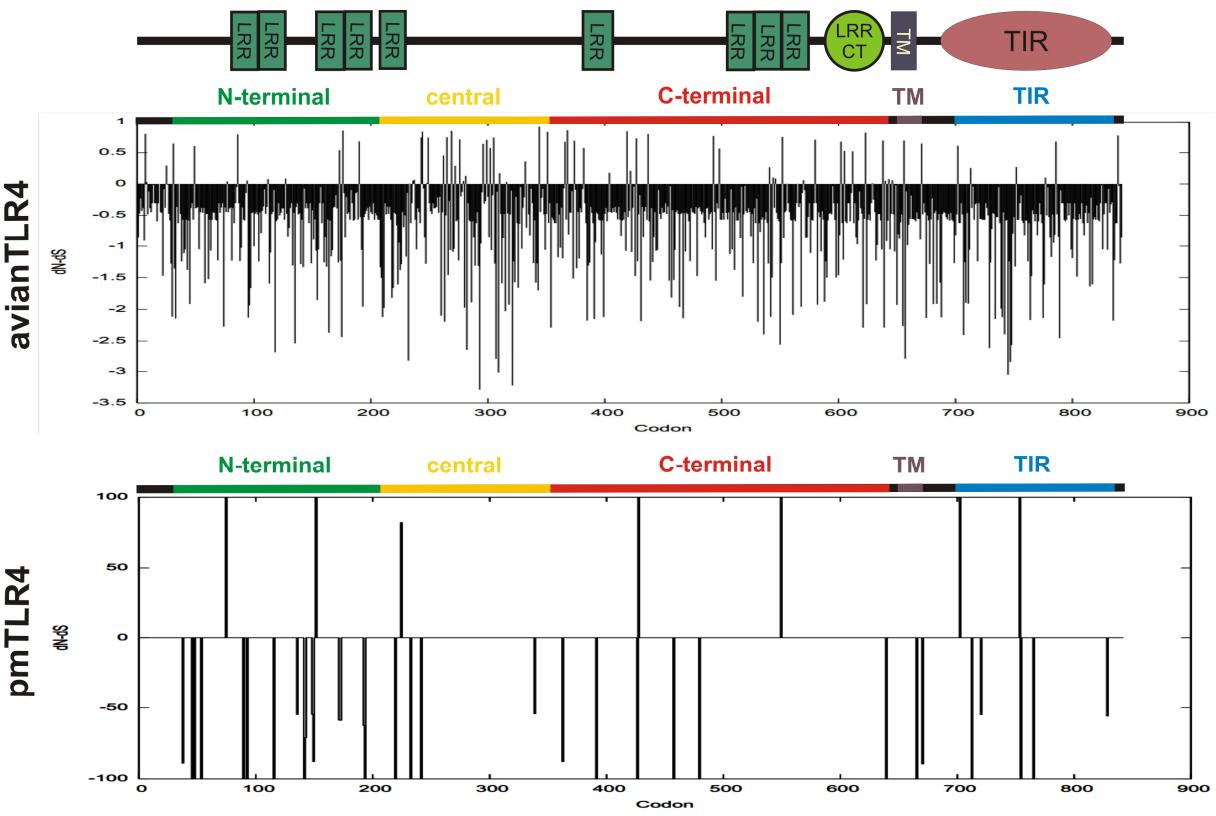


Fig.7 Selective sites in graphs with the respect to domains which might be expose to different selective pressures. The dN/dS (non-synonymous/synonymous) ratio is used to estimate positive selection at every codon in the alignments.

Tab.4 Interspecific sequence variability in TLR4 across passerine species; aa positives compared to tgTLR4 (ACN58232.1). N-terminal domain includes MD-2 binding site (TLR4-MD-2 complex is essential for LPS recognition).

	1-843 aa **TLR4**	31-206 aa **N-terminal**	207-351 aa **central**	352-640 aa **C-terminal**	644-666 aa **TM**	687-833 aa **TIR**
aaTLR4	94	95	89	95	100	97
ceTLR4	95	97	90	97	96	96
faTLR4	93	97	88	95	96	95
hrTLR4	93	96	88	94	96	95
lsTLR4	93	98	84	94	100	96
pdTLR4	94	97	90	94	100	96
pmTLR4	93	97	90	93	100	96
mean	**94**	**97**	**88**	**95**	**98**	**96**

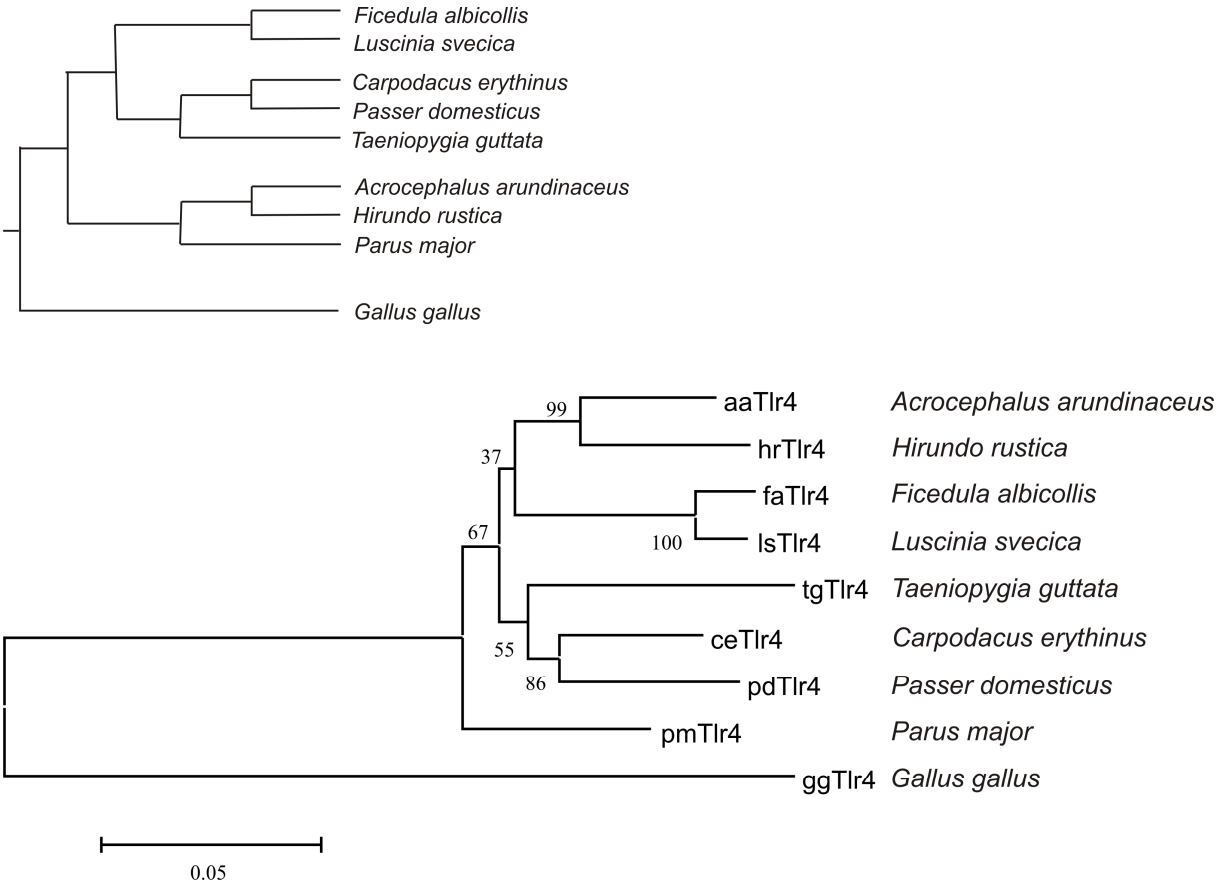


Fig.8 The passerines phenetic tree (lower) is not consistent with the known phylogenetic relationships in passerine birds based on several evolutionary neutral gene markers (modified according to Barker et al., 2002).

VIII.4. The influence of pmTLR4 polymorphism on condition and ornamentation

We have chosen 4 non-conservative amino acid substitutions, from all detected substitutions in pmTLR4, which were simultaneously very common in the population. The analysis of association between phenotypic traits in great tits and the genotypic polymorphism in TLR4 did not show any strong effect of any of the investigated common polymorphic sites in the gene. However, it has been revealed a marginally significant effect of the substitution Q549R on both melanin-based ornamentation (breast stripe area, P = 0.050, for MAM, see Tab.5 and Fig.9) and carotenoid-based ornamentation (plumage brightness, MAM: Q549R, df = 1/ 46, F = 4.15, P = 0.047; Fig.10). The breast stripe area and yellow plumage brightness were found to be independent traits (Pearson's product-moment correlation, r = 0.18, t = 1.23, df = 46, P = 0.224).

Tab.5 MAM indicating the association between breast stripe area and common non-synonymous SNPs in pmTLR4; n=48, df=2/45, F=40.44, P<<0.001

	Df	F	P
Sex	1	80.75	<< 0.001
Q549R	1	4.08	0.05

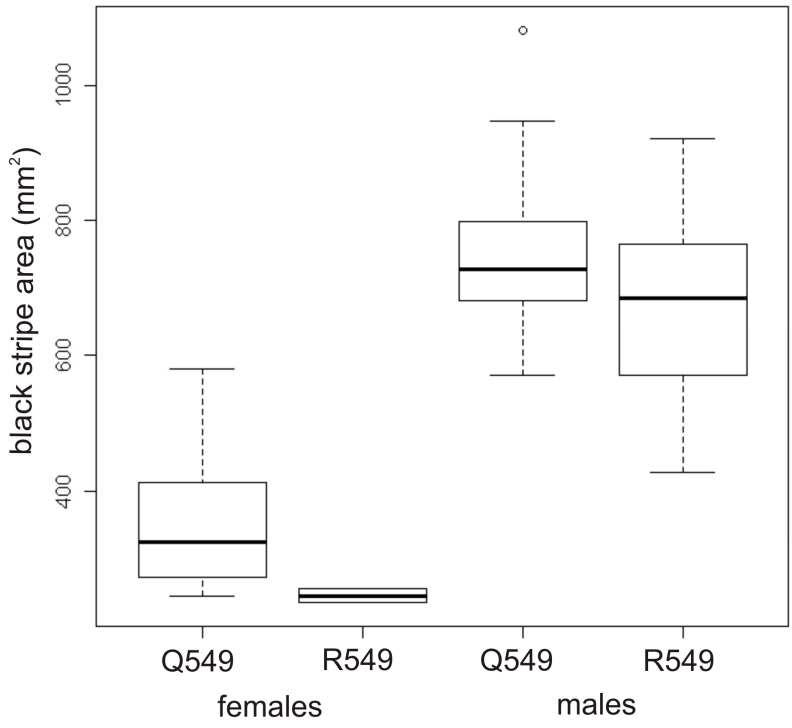


Fig.9 The significant relationship between melanin-based breast stripe area and bearing the certain amino acid was independent on sex. Individuals bearing R549 possess narrower stripe.

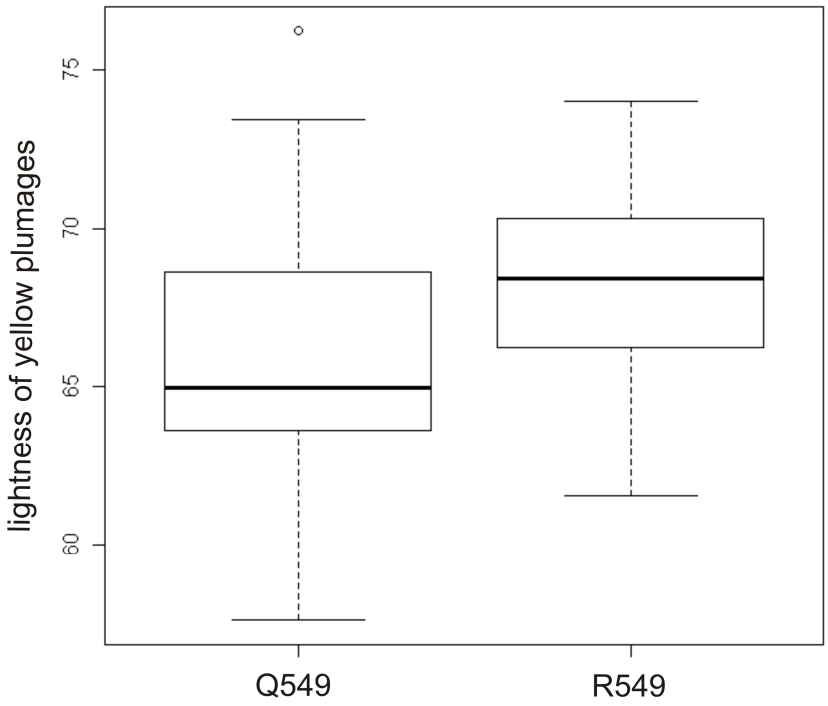


Fig.10 The significant relationship was found also between carotenoid-based breast yellow plumages (especially with lightness). More light plumage indicates feathers in worse condition (due to mites and other parasites). Individuals bearing R549 might be therefore in worst condition.

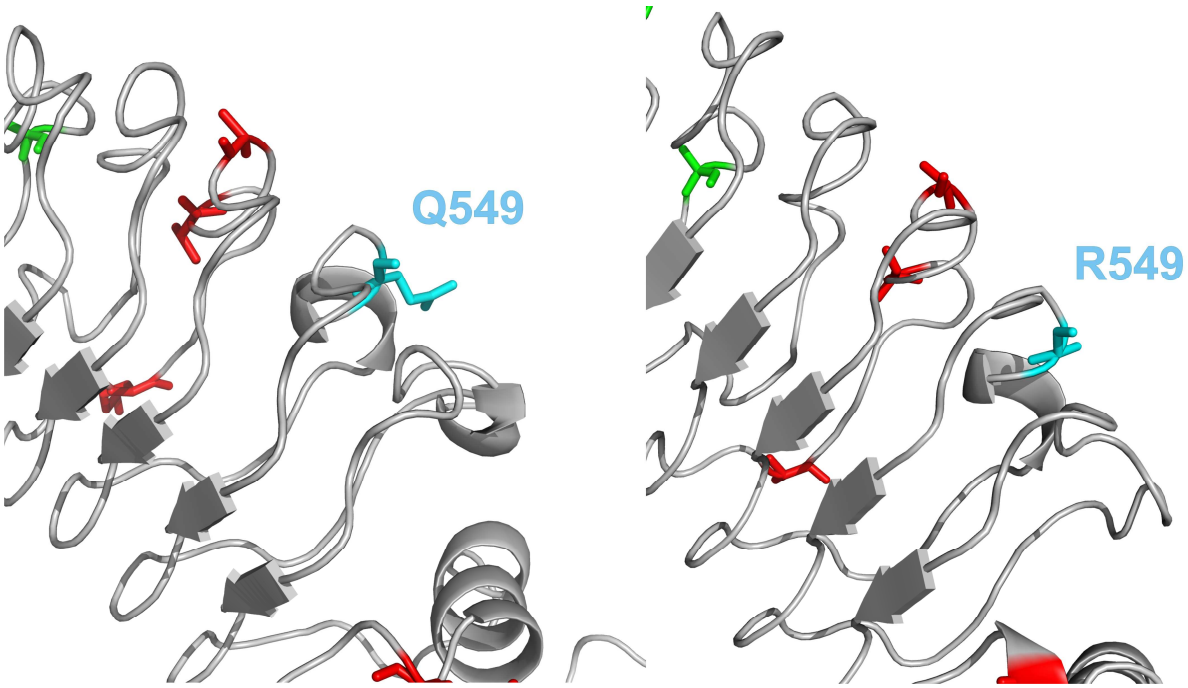


Fig. 11 The crucial substitution in blue highlighted. Based on this predicted structure, it seems that this substitution might significantly influence the function of receptor. Q549 has longer residue to the space while R549 structurally vapid. These amino acids are different alsoo accordance to their amino acid propeties.

IX. Discussion

In this diploma thesis I provide description of *Tlr4* gene in great tit and estimate level of its polymorphism in a free-living population of this species. To integrate these findings in *pmTlr4* into a broader evolutionary context we determined the *Tlr4* sequence also in other chosen passerine species. These data were then used for protein structure prediction. In passerines the TLR4 protein seems to show a high degree of structural similarity. In the same time, however, we revealed an extreme rate of genetic variability on both inter- and intra-specific level. In fact, each sampled individual of great tit was detected to be a heterozygote. In total we found 82 alleles in *pmTlr4* and 42 non-synonymous pmTLR4 haplotypes. Both synonymous and non-synonymous substitutions were found across the whole gene and positively selected sites were predicted to occur in extracellular as well as in intracellular domain. Results of phenotype-association analysis indicate that in great tit there might be a linkage between certain TLR4 amino acid substitutions and individual colouration. To my knowledge, this is the first study in free-living birds investigating the impact of TLR polymorphism on condition and ornamentation.

IX.1. TLR4 structure in great tit and other passerines

The whole TLR4 protein seems to be conservative as the shared similarity between phylogenetic distant species (including mammals) is high (more than 40%). In all passerines investigated the total length of the *Tlr4* coding sequence of all three exons was conserved, suggesting that also the protein amino acid length remains identical. When compared to ggTLR4, mmTLR4 and hsTLR4 one amino acid deletion arose in the passerine evolutionary linage (N245 in ggTLR4). The predicted tertiary structure of pmTLR4 shows similar horseshoe-like shape as the one found in crystalographically determined structures of mammalian TLR4 (Kim et al. 2007; Park et al. 2009). The predicted domain composition (LRRs, LRRCT, TM, TIR) remains very similar in all passerine TLR4. The only major difference between passerine and chicken TLR4 is in the absence of one LRR motive at the position 207-229 in ggTLR4 (Fig.5 ; see also Vinkler et al., 2009). Hence, the number of LRR motives that seem to form the structural backbone of the TLR4 protein (Kim et al. 2007) is not equal in all birds and even more of these differences can be found between birds and mammals. The LRRs are often presented as structures responsible for pathogen recognition regions (Akira et al. 2006). Different number and position of LRR motives might therefore

indicate dissimilar parasite pressures in evolution of birds and mammals. It has to be, however, noted that the direct involvement of TLR4 LRRs in ligand binding remains highly uncertain (Park et al. 2009). In all sequences a signal peptide which is crucial for successful guiding of a protein into cell membrane was detected and the cleavage site of the signal peptide was predicted at very similar positions in all passerine species sequenced.

IX.2. Evolution in passerine TLR4

Only a decade ago it was thought that genes acting in innate immunity should be more conservative than genes of the adaptive arm of immunity. This was because of the very ancient evolutionary origin of innate immunity (Medzhitov & Janeway 2000b; Rich 2005). Contrary to this former view, TLR4 seems to be extremely polymorphic in its amino acid sequence both among species and within them. Interspecific comparison of passerine TLR4 reveals relatively high level of sequence and structural similarity as well as many species-specific differences. This might be because the overall similarity is necessary for keeping suitable binding features to different kinds of PAMPs (e.g. LPS from Gram-negative bacterial cells in this case) while in the same time specific adaptations are required to improve the immune defence in a particular pathogen context. We can, therefore, see different trends in different regions of passerine TLR4. Most conservative seems to be the N-terminal part of the extracellular domain (probably because it includes MD-2 binding region; Kim et al. 2007; Park et al. 2009) and the intracellular TIR domain (probably due to its signalling function; Akira & Takeda 2004). On the other hand, most variable is the central part of the extracellular domain of the receptor. This may probably arise from varying parasite pressures on the extracellular domain among species. In the central part of this domain specific positions responsible for direct ligand binding are present. This corresponds with findings previously published in humans (Hajjar et al. 2002). In concordance with this explanation is also the outcome of the selection REL analysis that revealed positively selected sites in birds mostly in extracellular domain of TLR4 (especially in its central part) while no positive selection was detected in the TIR domain (Fig.7). This trend was previously reported in vertebrates also by (Smirnova et al. 2000; Hajjar et al. 2002; Hughes & Piontkivska 2008; Nakajima et al. 2008; Vinkler et al. 2009). However, it needs to be mentioned that although the REL analysis suits best to predictions of positively selected sites in small number of tested sequences, it also tends to report a relatively high number of false positives. These results should therefore be treated with some caution until larger dataset is collected.

Consistent with the previously reported findings are also the structure predictions suggesting that passerines share with other species the secondary structure of the intracellular TIR domain, in contrast to many regions in the extracellular domain that are far more variable. Also this could be explained by a crucial role of the TIR domain in signal transduction to a conservative signalling pathway leading to NF-κB (Akira & Takeda 2004). The most terminal part of the intracellular domain (last ten residues), however, seems to be highly variable in its secondary structure (some species possess in this region alpha-helixes and others beta sheets, see S5 in supplementary data). Even more variability in secondary structure can be found in the central part of the TLR4 extracellular domain. Although the analysis of recombination revealed two breakpoints in *Tlr4* sequence, which is consistent with independent evolution of different parts of the TLR4 molecule, these breakpoints do not directly match with N-terminal, central, C-terminal regions or any other structurally or functionally important boundaries.

In our dataset comparing interspecific variability in avian TLR4 we have found both conservative and variable positions in the residues that have been previously identified as important for the TLR4 function in hsTLR4 and mmTLR4 (Park et al. 2009; Kim et al. 2007). Nine amino acid positions were strictly conserved both in birds and mammals. These were residues mostly involved in TLR4-MD-2 interactions (C33, C44, D88, E139, H163, R238 counted according to pmTLR4) and TLR4-MD-2 dimerization (S424, L452). However, two of these highly conservative sites were also partially responsible for LPS-binding (S396, L452). It may be proposed that these sites are under strong purifying selection due to evolutionary stability of their counterparts. Seven other sites were distinct between birds and mammals and again these represented some of the predicted LPS-binding sites (Y448F, S471F) as well as positions responsible for interaction between TLR4 and MD-2 (V293R) or for TLR4-MD-2 dimerization (S447E, Y448F, S471F). The region between positions 28-51 which was described to form one of the MD-2 binding sites in hsTLR4 was found as generally conservative with most positions identical across all avian species and vast majority identical or at least similar by their biochemical features to their mammalian counterparts. Also in all these residues their high evolutionary stability may indicate the effect of purifying selection. On the contrary, five of the putatively important sites were revealed polymorphic. Four of these sites take part in LPS binding (267, 344, 368, 444) and only one in TLR4-MD-2-dimerization (425). From this comparison it seems that the variability in LPS-binding sites is much higher than variability in MD-2-binding sites. This indicates that direct TLR4-LPS

bonds might be under species-specific positive selection and may play an important role in host-parasite evolutionary interactions. Residue 471 is in hsTLR4 involved in both LPS and MD-2 binding. The dual selection pressure might be in this case responsible for the identically shared residue in all birds. Furthermore, none of the species-polymorphic sites was found variable in pmTLR4, which suggests that there might be different kinds of parasites among species selecting for the interspecific variability. Another possibility, however, is that the LPS binding is not dependent on specific features of the residues involved in the interaction. This seems to be unlikely at least in some cases given the physical and chemical features of the residues at the substitution sites and also given the limited consistence of the substitution occurrence with phylogenetic tree of passerine birds (compare Fig.8 with Barker et al., 2002). Finally, it is also possible that the residues involved in LPS binding in birds are different to those found in hsTLR4. Larger sample of species and more detailed immunological studies are needed to allow any final conclusions in this matter.

IX.3. Population polymorphism in pmTLR4

In our free-living great tit population we revealed about two times more SNPs than was previously revealed in any laboratory animal breeds (Smirnova et al. 2000; Leveque et al. 2003) or livestock (Downing et al. 2010; Jungi et al. 2011). The number of pmTLR4 alleles predicted in our great tit population was really high (82 alleles in 50 individuals), each individual carrying combination of two different alleles. The majority of alleles were rare, mostly detected only once in the study population. Similar situation was also in the case of non-synonymous haplotypes. Interestingly we found that these minor haplotypes do not co-occur in heterozygote genotypes with other minor haplotypes. The minor haplotypes are always present together with one of the common haplotypes. This might indicate detrimental effect of these minor haplotypes and lower relative fitness of individuals carrying two recessive alleles. Nevertheless, statistical analysis showed insignificance of the relationship; the observed minor haplotype frequencies corresponded to the expected haplotype frequencies under the Hardy-Weinberg equilibrium.

Polymorphic sites were distributed along the whole gene length (see S3 in supplementary data). Thus, there was no evidence for a particular region accumulating most SNPs. Despite this there were differences in substitution composition in individual protein regions. In average, synonymous SNPs were more abundant than non-synonymous SNPs. However, in LRR motives the proportion of synonymous to non-synonymous SNPs was

found to be marginally significant. This might be explained by purifying selection acting on non-LRR regions, by lack of purifying selection in LRRs or by positive selection in LRRs. Potentially, this variation might be related to pathogen detection. Some of the LRR-based non-synonymous substitutions were previously reported in chickens (Y383H and Q611R; Leveque et al. 2003) and in humans (D299G and T399I; Kiechl et al. 2002; Ferwerda et al. 2007; Misch & Hawn 2008) to influence susceptibility to diseases.

Undoubtedly, more influential are non-conservative amino acid substitutions changing chemical or physical properties of the residue as these may substantially influence the protein function. Surprisingly, in pmTLR4 majority of detected amino acid substitutions were non-conservative with potential to change the local physicochemical properties in the protein (see S3 in supplementary data). For instance, the substitution E173K replaced negatively charged glutamid acid with positively charged basic lysin. This position is interesting also based on the interspecific comparison in birds, because the same polymorphism as in great tits occurs also in bluethroats and in house sparrows (see S4 in supplementary data). Other passerines possess either E (great reed warbler, collared flycatcher, barn swallow) or K (scarlet rosefinch, zebra finch) and in domestic chicken K173 may be replaced by a stop codon (Downing et al. 2010).

Out of the non-conservative non-synonymous substitutions only few were located at positions with known impact on the binding performance of the protein in model species. PmTLR4 non-conservative amino acid substitution, K342Q, is located structurally very closely to the direct LPS-binding site in hsTLR4 (pmTLR4 position 344/hsTLR4 position 341; Park et al. 2009). The bond of microbe-derived LPS to TLR4 molecule enables dimerization of TLR-MD2 heterodimers which is responsible for signal transduction (Kim et al. 2007). The K342Q substitution might hence influence binding specificity of TLR4 to LPS and change the pathogen recognition ability of the cell. In great tit population the Q342 variant is rare and we may speculate that this might be due to current negative selection to this substitution. This allele might, nevertheless, represent a source of variability that would be positively selected under altered conditions of the pathogen environment (cyclic selection, selection fluctuating in time and space; Hamilton & Zuk 1982).

In addition to interaction with LPS, the TLR4 molecule also needs to bind the MD-2 molecule. Amino acid substitutions occurring at the MD-2-binding region may potentially alter the TLR4-MD-2 interaction and thus change the conformation of the LPS binding

pocket. A frequently occurring substitution T427A is located closely to one of the MD-2-binding residues identified in hsTLR4 (pmTLR4 position 344/hsTLR4 position 341; Park et al. 2009). Nevertheless, it seems that this particular substitution may not have any strong effect on receptor function because of its frequency.

Two frequent non-conservative amino acid substitutions were detected also in the intracellular TIR domain of pmTLR4 (see S2 in supplementary data). This is remarkable, as the TIR domain was repeatedly shown to be under reasonably strong purifying selection (Smirnova et al. 2000; see also above). This conservativeness of the TIR domain seems to be mainly due to its role in signalling function of the protein. The amino acid substitution in this region might, hence, strongly affect the signalling outcome of the LPS binding and alter the immunological defence against parasites.

Consistent with these findings are also the results of the IFEL selection analysis that shown in pmTLR4 two out of four positively selected sites in the TIR domain. No negatively selected site was found in the highly variable central part of the extracellular domain, which is in accordance with released negative selection acting on this part of TLR4. Positively selected sites detected by Alcaide and Edwards (Alcaide & Edwards 2011) in several bird species are inconsistent with sites revealed under positive selection in our study. The only exception was position 351 (counted according to pmTLR4). But we are not able to obtain information about large part of gene from this study because the authors presented only a part of the gene and there is also no notation about negatively selected sites. Despite, it might suggest that the central part of extracellular domain of TLR4 is the possible target of positive Darwinian selection in the course of birds evolution.

IX.4. The influence of pmTLR4 polymorphism on condition and ornamentation

In our great tit population we found reasonably high frequencies in four non-conservative amino acid substitutions. Of these substitutions none was related to morphological condition-dependent traits such as body mass, size or mean feather growth bar width. No association was also revealed between these four substitution sites and peripheral blood leukocyte count, a health-indicating marker. However, a significant linkage was found between substitution Q549R and plumage ornamental colouration (see Fig.9 and Fig.10). Namely, our results indicate that there might be a relationship between certain amino acid at

this position in pmTLR4 and lightness of yellow plumage and size of black stripe. In both sexes individuals bearing arginine (R549) expressed narrower melanin-pigmented black breast stripes and lighter carotenoid-pigmented yellow breast colouration then birds with glutamine (Q549) in both alleles (see Fig.11). There was, however, no correlation between feather lightness and breast stripe area. In the breast stripe area, there was also a significant effect of sex in the interaction. This is because males and females in great tits show high levels of sexual dichromatism in this trait (Cramp et al. 1994).

Our results correspond to other studies that map the impact of TLR4 variability on susceptibility to diseases. In most of these studies only several polymorphic sites in the gene are responsible for the immunological profile of the individual and thus also for the disease resistance (Arbour et al. 2000; Kiechl et al. 2002; Chen et al. 2005; Swiderek et al. 2006; Ferwerda et al. 2007; Mucha et al. 2009).

The relationship between polymorphism in IRGs (mostly the rate of heterozygosity) and sexual selection is well known in MHC genes (reviewed in Milinski, 2006). Most evidence demonstrates that maximal or optimal levels of the MHC heterozygosity are related to lower parasite burden (Westerdahl et al. 2005) and more elaborated ornamentation (von Schantz et al. 1996). But the relationship between IRG polymorphism and bird colouration is still not so well support. Nevertheless, several studies report an interaction of this kind with MHC in fish, reptiles and mammals (Olsson et al. 2005; Jager et al. 2007; Setchell et al. 2009).

In this study we found the first evidence for possible association between TLR polymorphism and ornamental colouration in animals. In general, our data show influence of innate immunity on ornamental signalling and support indicator model of sexual selection. In great tit the ornamental plumage colouration represents a health- and condition-related trait (Horak et al. 1999; Horak et al. 2000; Senar et al. 2003). Both melanin-based and carotenoid-based ornamentation is involved in sexual selection in this species, with wider melanin-pigmented stripe and yellower carotenoid-pigmented plumage being the preferred states of the traits (Norris 1990; Kingma et al. 2008). Melanin-based ornamentation represented in great tits mainly by the area of the black breast stripe is strongly related to dominance and social status of the individual (Jarvi & Bakken 1984; Quesada & Senar 2007). However, also this type of ornamentation as well as the carotenoid-based colouration was found in association with individual health (Dufva & Allander 1995; Horak et al. 2001). The yellow colouration

possesses three components (hue, saturation and lightness) and each of these features indicates a special aspect of the colourful signal. In our dataset, correlation was revealed only between yellow feather lightness and pmTLR4 polymorphism. This might indicate that rather the rate of feather damage and not the plumage carotenoid content is influenced by the substitution. As in the case of bluebirds (Shawkey et al. 2007) the feather damage may be in tits caused by ectoparasites. In any case, both narrower breast stripe and lighter yellow plumage seem to indicate worsen individual quality. The R549 variant might be therefore responsible for decrease in individual quality. This might be due to conformation changes in the receptor causing its altered ligand binding features. It is possible that the Q549 variant is better adapted to detection of currently and locally most common parasite variants. This would be supported also by the lower incidence of the R549 variant in the great tit population. Although no association of this variant to condition- or health-related traits was revealed this might be only because of the nebulous nature of condition and health (Hill 2011). It is likely that the putative condition- and health-related traits were in fact unimportant or insufficient to explain differences in condition and health differences in our study population. This is, nonetheless, not the only possible explanation of this linkage and as the significance level of the relationship between Q549R polymorphism and ornamental colouration obtained in this study is rather low, this linkage should be treated with some caution before it is verified in a larger dataset.

X. Conclusion

Innate immunity forms an arm of immune system which is of at least equal importance to antiparasite defence as adaptive immunity. It provides the first wave of host immunological protection against various potential parasites, either by direct triggering of effector immunological mechanisms or by co-stimulation of adaptive immunity. Therefore, its optimal function is crucial to host survival and individuals bearing immunological genotypes better adapted to the actual ecological context are expected to obtain higher fitness. TLRs are among the first receptors of the vertebrate immune system to sense pathogen incursion into the host organism. It has been well established that their optimal responsiveness is essential for successful defence against various parasites. TLRs evolve through frequent interactions with specific PAMP structures. As these structures are polymorphic in parasites, this interaction might maintain also polymorphism in the target receptor. Moreover, changes in occurrence of various parasite types, species and strains in time and space may cause fluctuations in selection pressures posing of TLRs. This co-evolutionary arms race might be tracked in the TLR structure. The same what concerns TLRs in general seems to be true also for TLR4 in particular. In TLR4 this idea of host-parasite co-evolution was supported by dozens of medical, veterinary and immunological studies presenting various relationships between polymorphic sites in TLR4 and susceptibility to inflammatory and infectious diseases.

In my diploma thesis I report the first description of complete *Tlr4* translated region and prediction of the TLR4 protein structure in non-model free-living passerine birds. In great tit we assessed population level of TLR4 polymorphism. We revealed similar protein structures but simultaneously an extreme rate of genetic variability in passerine TLR4 receptors. On interspecific level SNPs were found across the whole gene but most positively selected sites were detected in the extracellular domain which is responsible for pathogen recognition. Similarly, also within the great tit population SNPs were found across the whole gene but non-synonymous substitutions were present mainly in LRRs. Nevertheless, positively selected sites were in pmTLR4 detected in both extracellular and intracellular domain. In total, we revealed 82 alleles in *pmTlr4* and 42 non-synonymous pmTLR4 haplotypes. Most importantly, in great tits we found a relationship between a TLR4 SNP variant and condition-related traits. The crucial amino acid substitution, Q549R, is associated with elaboration of two independent traits in the plumage ornamentation. Individuals carrying R549 variant express narrower melanin-pigmented black breast stripe and lighter carotenoid-

pigmented yellow breast colouration. As both these traits seem to indicate worsen individual quality it seems that the substitution might influence host fitness in natural as well as sexual selection. Several explanations may be applied to the linkage between Q549R polymorphism and ornamental colouration. The most probable one is that this amino acid substitution may cause a conformation change in the receptor and thus alter its ligand binding features. The Q549 variant might be then better adapted to recognise currently and locally most common great tit parasites. To my knowledge, this is the first study in free-living birds which investigated the possible linkage between polymorphism in TLRs and condition-dependent ornamentation in animals. These findings are in accordance with the indicator model of sexual selection.

Future work in this model system should cover the investigation of the association of TLR4-LPS with other members of the LPS binding complex. The research should focus on the description of structure and level of population polymorphism in LBP, CD14, MD-2 as LPS binding molecules and MyD88 as the key signalling molecule coming into an physical interaction with TLR4 TIR domain. Relationships found between polymorphism in these molecules and condition-dependent traits (such as plumage ornamentation) in non-model free-living birds should be verified by in vitro functional analyses in cells carrying distinct SNP variants. As a result complex picture of the ecology and evolution of immunity pathogen recognition in free-living birds might be obtained. However, even if this work is undertaken we are still at the very beginning in gathering all relevant information to this topic. TLRs form only a small part of the immunological defence which remains (perhaps apart of MHC) unexplored. Hence, completely novel field of ecological, immunological and evolutionary investigation is being opened and it will be interesting to learn how variability in different IRGs co-influence the hosts defence against various parasites.

XI. Acknowledgement

First of all I would like to acknowledge to my supervisor Michal Vinkler especially because he gave me a chance to try penetrate into various immune mechanisms with the accent to immune function in natural and sexual selection and this is real challenge for me. I thank Michal once more for his valuable and stimulating suggestions throughout writing my thesis, I believe to utilize them in a future. My special thanks belong to Marta Promerová and Aňa Bryjová to provide me the best laboratory basics and no less to all the people from Studenec to make fantastic athmosphere. I would like to appreciate the help from Jitka Poplová, Martina Soudková, Pavel Pipek, Ondra Šťovíček and Tereza Kubíková with the data collection in the field. I thank also Tomáš Albrecht for statistical analyses, Josef Bryja for generating the phenetic tree and against Michal Vinkler for providing me the data on the carotenoid-based ornamentation colouration and leukocytes count. Great thanks belong to my parents and Honza for continual support and that they trust me.

This work was supported by the Czech Science Foundation (project GAČR P505/10/1871) and the Grant Agency of Charles University (projects GA UK No. 127507 and No. 44809).

XII. Dictionary of abbreviations

CD14	Cluster of differentiation 14
IRG	Imunity related gene
LBP	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LRR-CT	Leucine-rich repeat - carboxy terminal
MAM	Minimum adequate model
MHC	Major Histocompatibility Complex
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
Th	T-helper
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor

XIII. References

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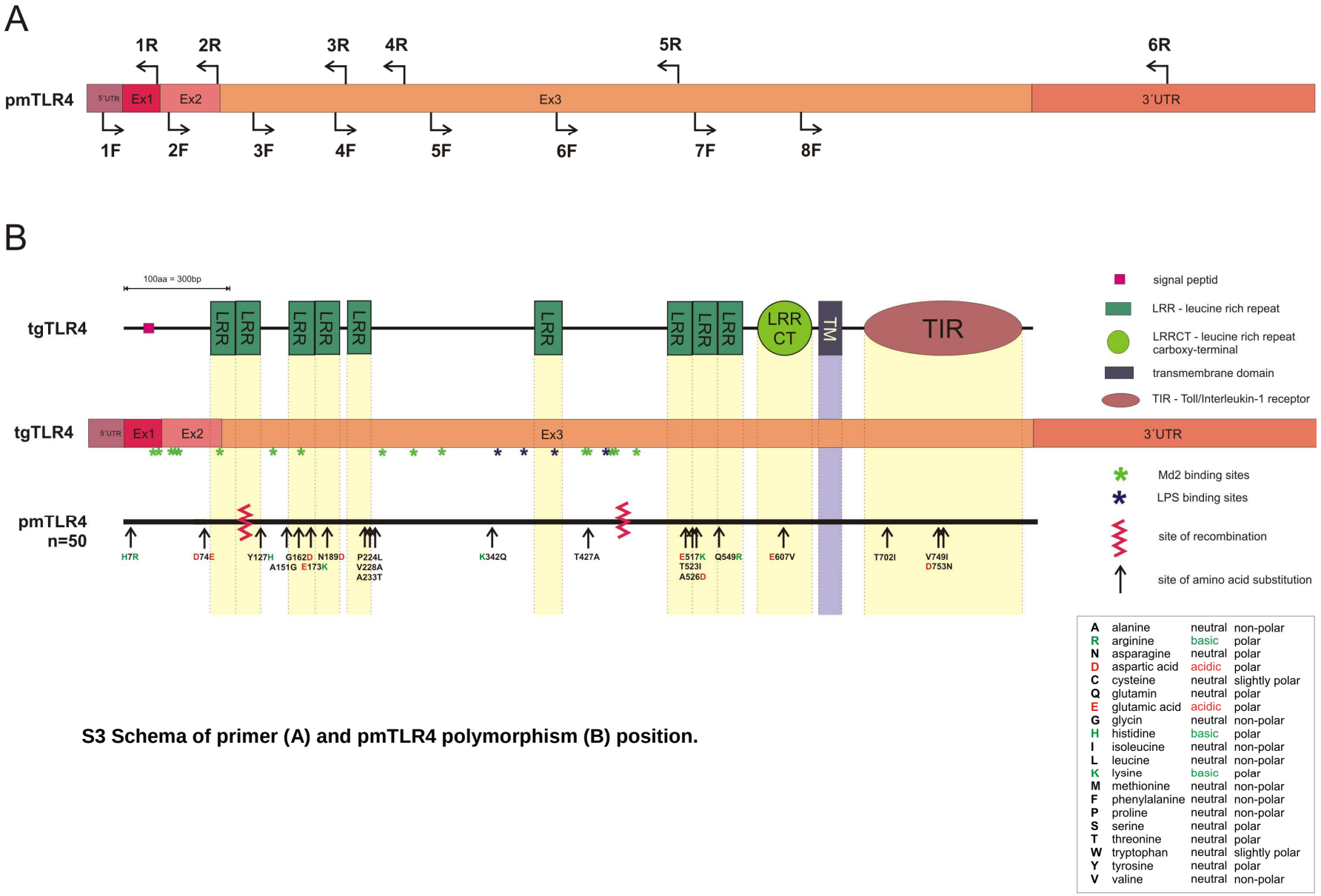
XIV. Supplementary data

S1 PmTLR polymorphism detected in 50 randomly chosen individuals. Position of substitutions in the nucleotide (in bp) and amino acid (in aa) sequence starting with the initial ATG (methionine). SS indicates synonymous substitution, NS indicates non-synonymous substitution (highlighted also in bold).

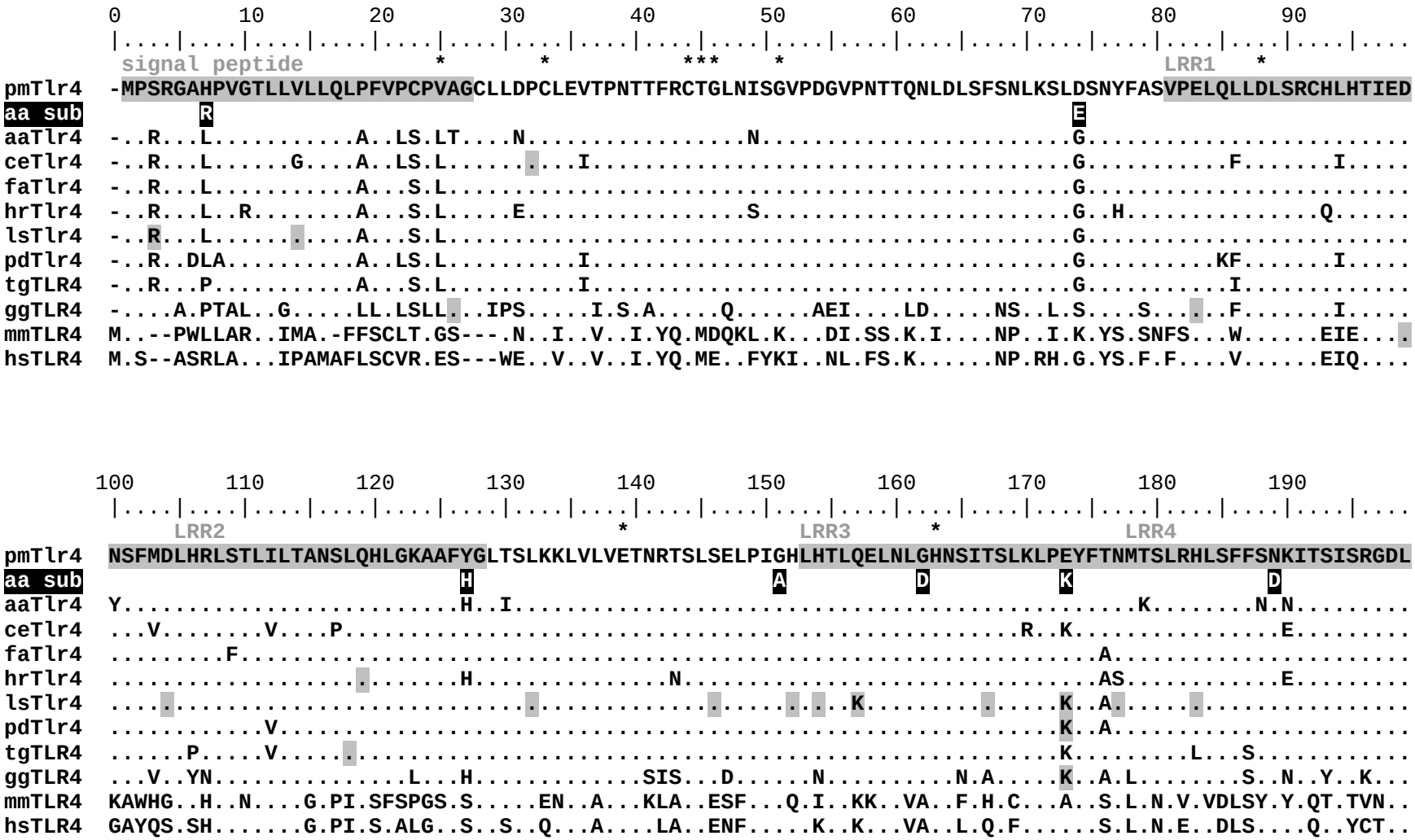
bp	aa	triplet	mutation	aa substitution
20	**7**	**C(A/G)T**	**NS**	**H7R**
111	37	AC(C/T)	SS	
135	45	AC(T/C)	SS	
141	47	CT(G/C)	SS	
159	53	CC(T/C)	SS	
222	**74**	**GA(C/G)**	**NS**	**D74E**
267	89	CT(C/T)	SS	
276	92	CG(C/T)	SS	
345	115	GC(C/T)	SS	
379	**127**	**(T/C)AT**	**NS**	**Y127H**
403	135	(C/T)TG	SS	
423	141	AA(C/T)	SS	
426	142	AG(A/G)	SS	
444	148	CT(A/G)	SS	
447	149	CC(C/T)	SS	
452	**151**	**G(C/G)A**	**NS**	**A151G**
453	151	GC(A/C)	SS	
485	**162**	**G(A/G)C**	**NS**	**G162D**
513	171	CT(C/T)	SS	
516	172	CC(T/C)	SS	
517	**173**	**(G/A)AG**	**NS**	**E173K**
565	**189**	**(A/G)AC**	**NS**	**N189D**
576	192	AC(A/G)	SS	
579	193	TC(A/T)	SS	
657	219	AT(A/C)	SS	
671	**224**	**C(C/T)G**	**NS**	**P224L**
683	**228**	**G(C/T)G**	**NS**	**V228A**
696	232	CT(C/T)	SS	
697	**233**	**(A/G)CT**	**NS**	**A233T**
723	241	TT(T/C)	SS	
1014	338	CT(A/G)	SS	
1024	**342**	**(A/C)AA**	**NS**	**K342Q**
1086	362	CT(C/T)	SS	
1173	391	AA(C/T)	SS	
1278	426	TT(C/T)	SS	
1279	**427**	**(A/G)CT**	**NS**	**T427A**
1369	457	(C/T)TG	SS	
1437	479	AA(C/T)	SS	
1549	**517**	**(G/A)AA**	**NS**	**E517K**
1568	**523**	**A(C/T)T**	**NS**	**T523I**
1577	**526**	**G(A/C)C**	**NS**	**A526D**
1646	**549**	**C(A/G)G**	**NS**	**Q549R**
1820	**607**	**G(A/T)G**	**NS**	**E607V**
1917	639	AG(C/T)	SS	
1995	665	TT(C/T)	SS	
2010	670	TC(C/T)	SS	
2105	**702**	**A(C/T)A**	**NS**	**T702I**
2136	712	GG(A/G)	SS	
2160	720	CT(C/G)	SS	
2245	**749**	**(A/G)TC**	**NS**	**V749I**
2257	**753**	**(A/G)AC**	**NS**	**D753N**
2262	754	TT(C/T)	SS	
2295	765	GA(C/T)	SS	
2482	828	(C/T)TG	SS	

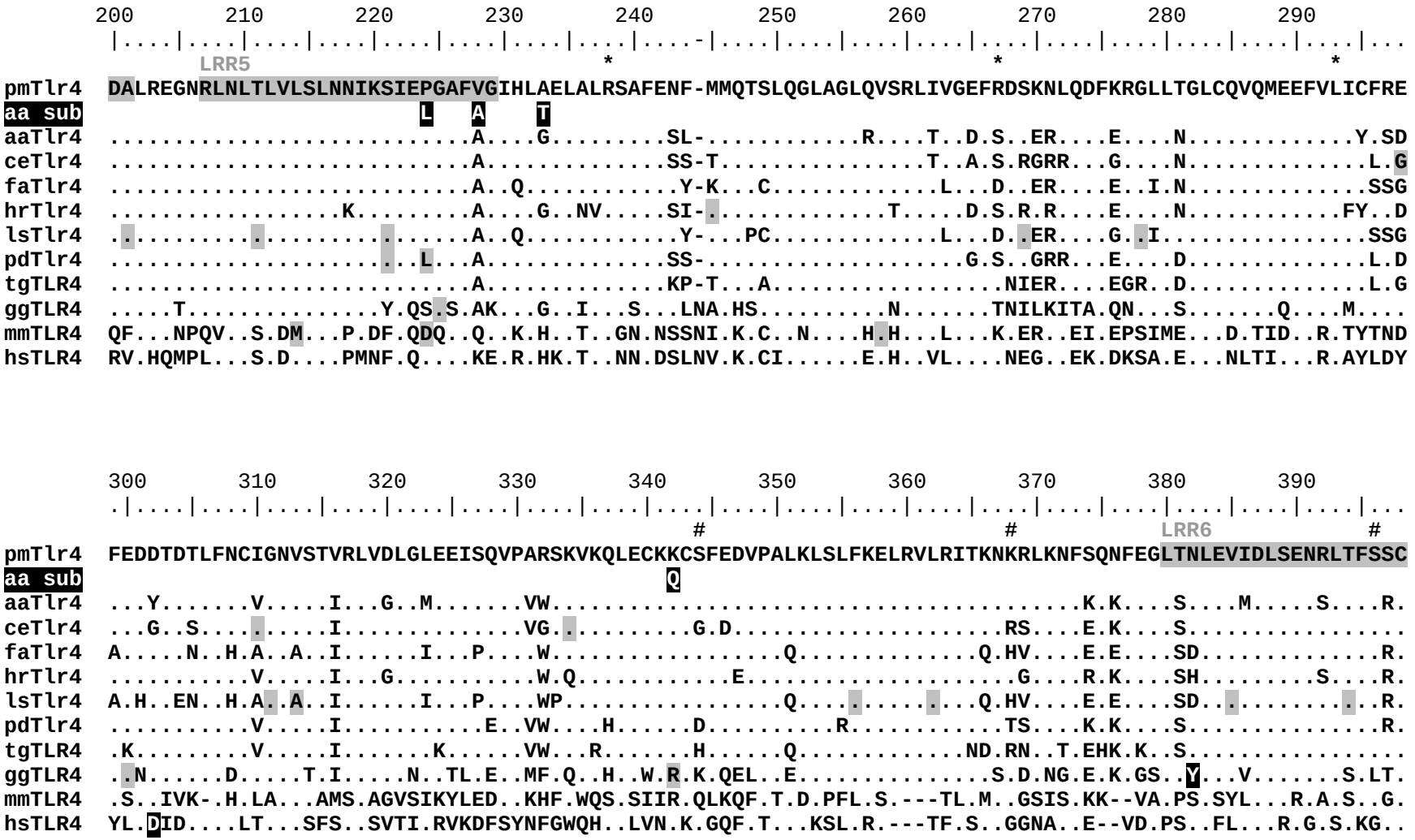
S2 Non-synonymous polymorphism in *pmTlr4*. Conservative substitutions are highlighted in italics, substitutions changing aa features are highlighted in by bolt. Position of each substitution within TLR4 domains and motives is given (N – N-terminal domain, central – central domain and C – C-terminal domain, TIR – TIR domain, LRR – LRR motive) and predicted involvement in secondary structure formation is indicated (H – alfa helix, E – beta sheet). To each NS site also the information on number of variant haplotypes and frequencies is provided. Frequent non-conservative substitutions are marked by black background colouration.

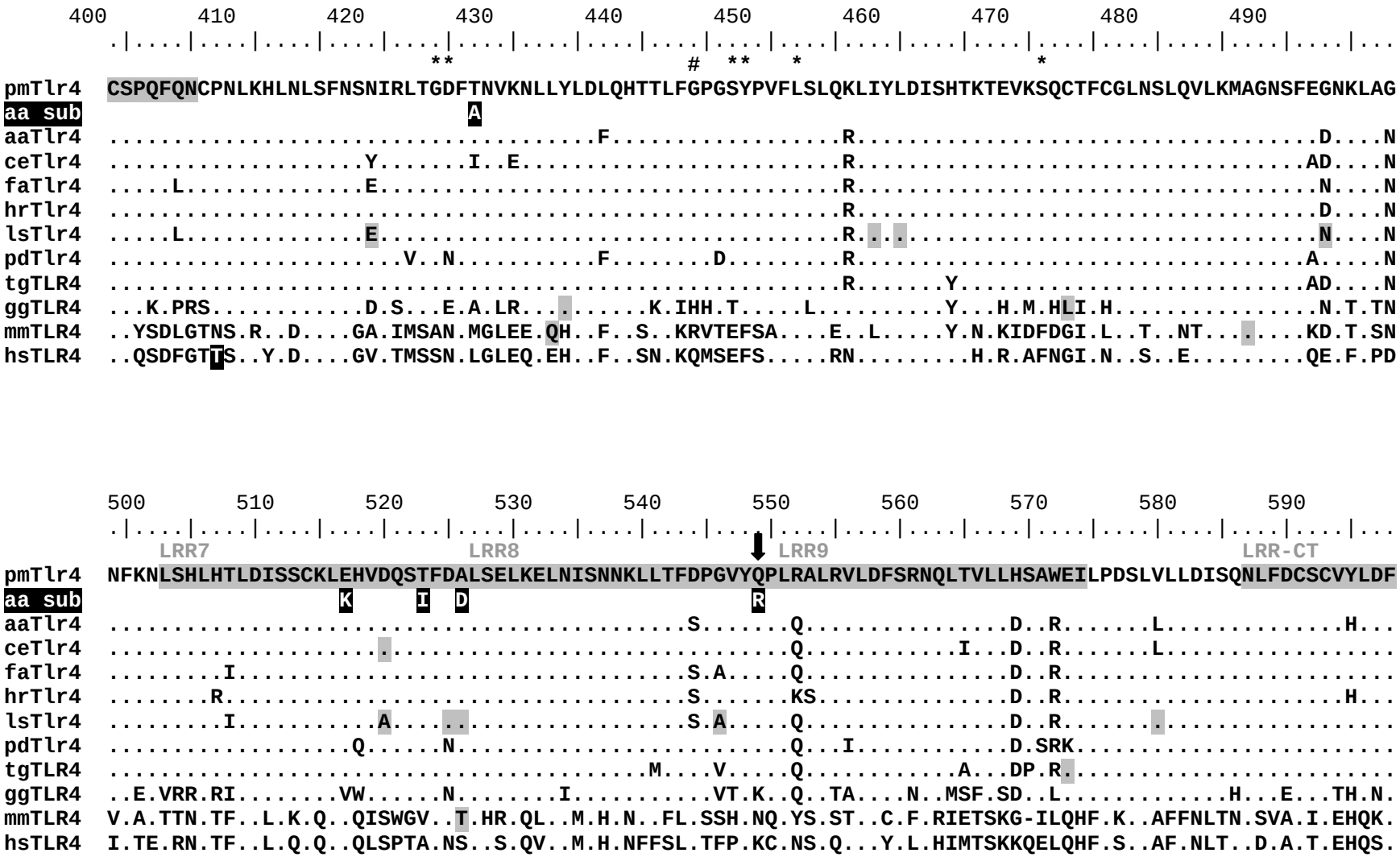
aa subst.	aa properties	Domains	Motives	2D	No. hapl. 1	No. hapl. 2	f 1 (%)	f 2 (%)
H7R	*= both positively charged*	*-*	*-*	*H*	*40*	*2*	*98*	*2*
D74E	*= both negatively charged*	*N*	*-*	*-*	*26*	*16*	*58*	*42*
Y127H	*= both aromatic*	*N*	*LRR2*	*-*	*41*	*1*	*99*	*1*
A151G	*= both small*	*N*	*-*	*H*	*29*	*13*	*80*	*20*
G162D	**? small › negatively charged**	**N**	**LRR3**	**-**	**41**	**1**	**99**	**1**
E173K	**? negatively charged › positively charged**	**N**	**LRR3**	**H**	**41**	**1**	**99**	**1**
N189D	**? NH2 without charge › negatively charged**	**N**	**LRR4**	**-**	**41**	**1**	**99**	**1**
P224L	**? unique › aliphatic hydrophobic**	**central**	**LRR5**	**-**	**40**	**2**	**97**	**3**
V228A	**? aliphatic hydrophobic › small**	**central**	**LRR5**	**-**	**40**	**2**	**98**	**2**
A233T	**? small › hydrophilic OH**	**central**	**-**	**-**	**39**	**3**	**97**	**3**
K342Q	**? positively charged › NH2 without charge**	**central**	**-**	**-**	**41**	**1**	**99**	**1**
T427A	**? hydrophilic OH › small**	**C**	**-**	**H**	**25**	**17**	**69**	**31**
E517K	**? negatively charged › positively charged**	**C**	**LRR7**	**-**	**39**	**3**	**97**	**3**
T523I	**? hydrophilic OH › hydrophobic aliphatic**	**C**	**LRR7**	**H**	**41**	**1**	**99**	**1**
A526D	**? small › negatively charged**	**C**	**LRR7**	**-**	**41**	**1**	**99**	**1**
Q549R	**? NH2 without charge › positively charged**	**C**	**LRR8**	**-**	**30**	**12**	**77**	**23**
E607V	**? negatively charged › aliphatic**	**C**	**LRRCT**	**H/E**	**41**	**1**	**99**	**1**
T702I	**? hydrophilic OH › hydrophobic aliphatic**	**TIR**	**-**	**H**	**26**	**16**	**69**	**31**
V749I	*= both hydrophobic aliphatic*	*TIR*	*-*	*E*	*39*	*3*	*97*	*3*
D753N	**? negatively charged › NH2 without charge**	**TIR**	**-**	**H**	**27**	**15**	**63**	**37**

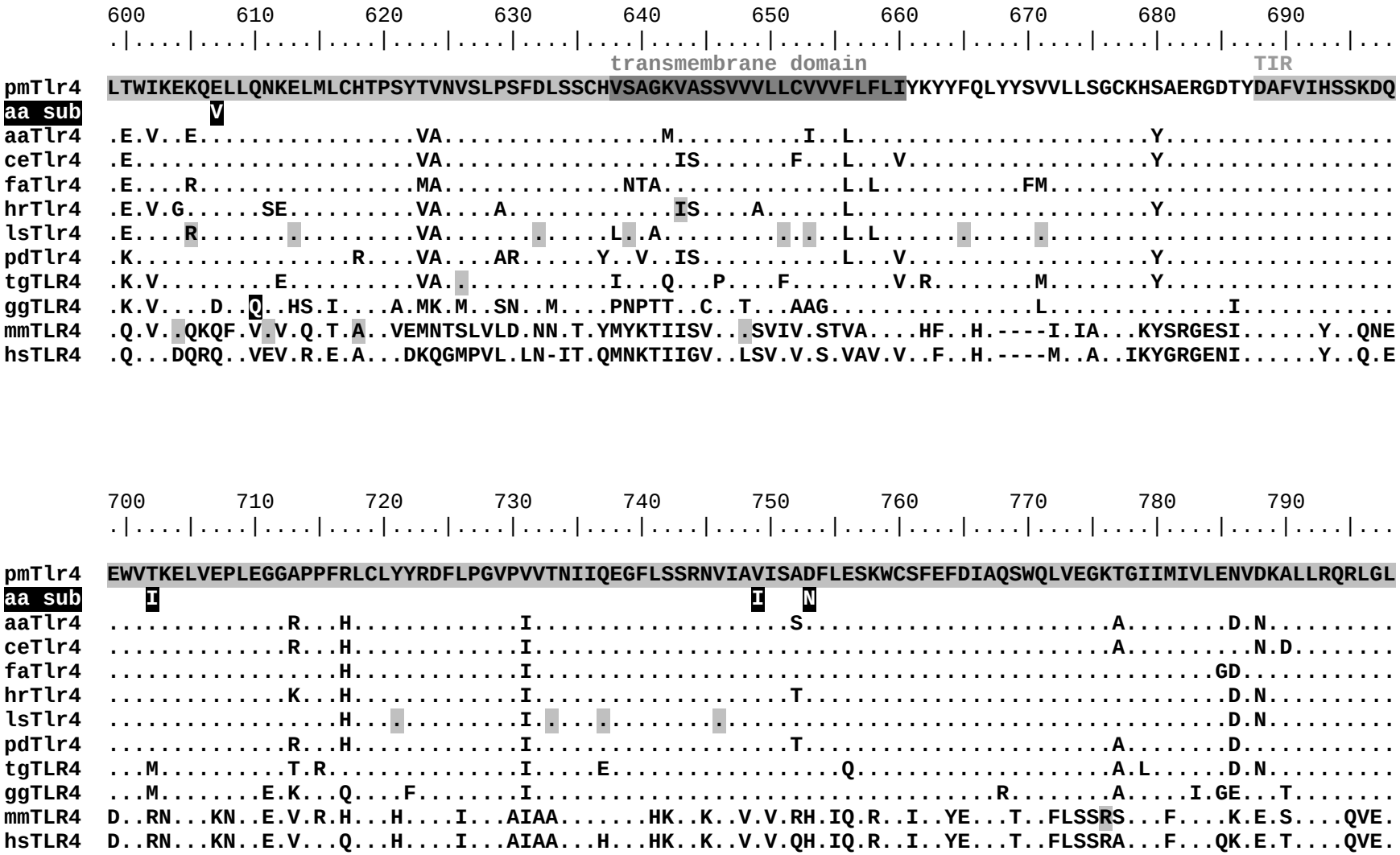


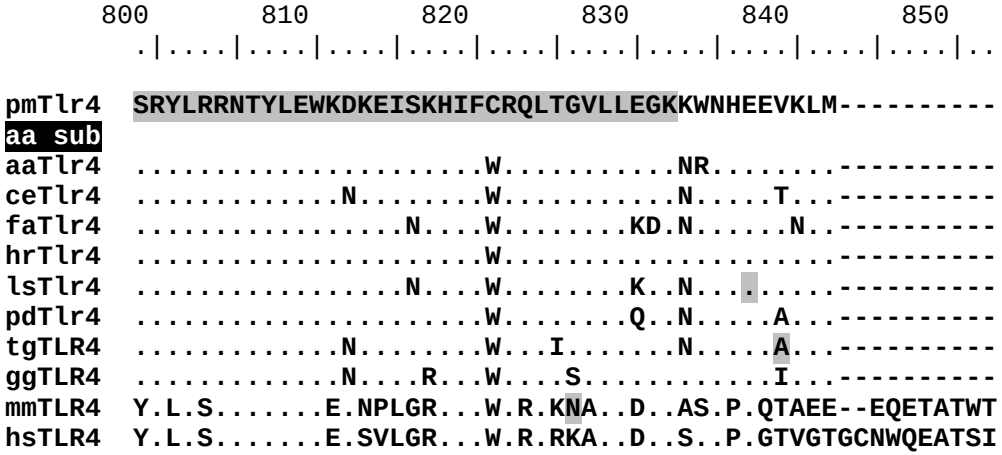
S3 Schema of primer (A) and pmTLR4 polymorphism (B) position.


0 10 20 30 40 50 60 70 80 90
signal peptide
LRR1
pmTlr4 -MPSRGAHPVGTLLVLLQLPFVPCPVAGCLLDPCLEVTPNTTFRCTGLNISGVPDGVPNTTQNLDLSFSNLKSLDSNYFASVPELQLLDLSRCHLHTIED
aa sub R E
aaTlr4 -..R...L.....A..LS.LT....N.....N.....G.....
ceTlr4 -..R...L......G....A..LS.L.....I.....G.....F.....I.....
faTlr4 -..R...L.....A...S.L.....G.....
hrTlr4 -..R...L..R.....A...S.L.....E.....S.....G..H.....Q.....
lsTlr4 -..R...L.....A...S.L.....G.....
pdTlr4 -...R..DLA.....A..LS.L.....I.....G.....KF.....I.....
tgTLR4 -...R...P.....A...S.L.....I.....G.....I.....
ggTLR4 -....A.PTAL..G.....LL.LSLL...IPS.....I.S.A.....Q.....AEI....LD.....NS..L.S....S.....F.....I.....
mmTLR4 M..--PWLLAR..IMA.-FFSCLT.GS---.N..I..V..I.YQ.MDQKL.K...DI.SS.K.I....NP..I.K.YS.SNFS...W.....EIE....
hsTLR4 M.S--ASRLA...IPAMAFLSCVR.ES---WE..V..V..I.YQ.ME..FYKI..NL.FS.K.....NP.RH.G.YS.F.F....V.....EIQ....
100 110 120 130 140 150 160 170 180 190
LRR2
LRR3
LRR4
pmTlr4 NSFMDLHRLSTLILTANSLQHLGKAAFYGLTSLKKLVLVETNRTSLSELPIGHLHTLQELNLGHNSITSLKLPEYFTNMTSLRHLSFFSNKITSISRGDL
aa sub H A D K D
aaTlr4 Y.....H..I.....K.....N.N.....
ceTlr4 ...V.....V....P.....R..K.....E.....
faTlr4 .....F.....A.....
hrTlr4 .....H.....N.....AS.....E.....
lsTlr4 .....K.....K..A.....
pdTlr4 .....V.....K..A.....
tgTLR4 .....P.....V.....K.....L...S.....
ggTLR4 ...V..YN.....L...H.....SIS...D.....N.....N.A.....K..A.L.....S..N..Y..K...
mmTLR4 KAWHG..H..N....G.PI.SFSPGS.S.....EN..A...KLA..ESF...Q.I..KK..VA..F.H.C...A..S.L.N.V.VDLSY.Y.QT.TVN..
hsTLR4 GAYQS.SH.....G.PI.S.ALG..S..S..Q...A....LA..ENF.....K..K...VA..L.Q.F.....S.L.N.E..DLS....Q..YCT..

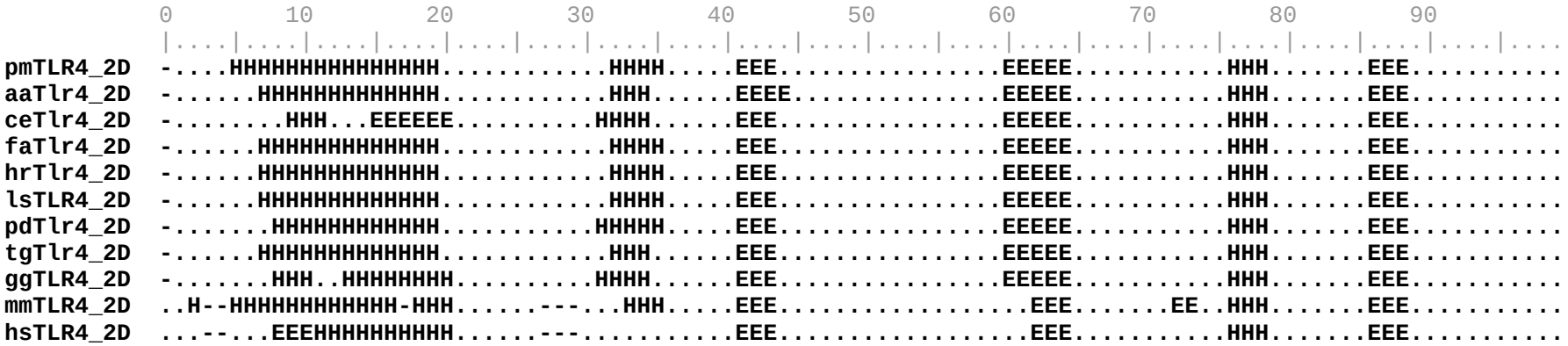

200 210 220 230 240 250 260 270 280 290
LRR5
pmTlr4 DALREGNRLNLTLVLSLNNIKSIEPGAFVGIHLAELALRSAFENF-MMQTSLQGLAGLQVSRLIVGEFRDSKNLQDFKRGLLTGLCQVQMEEFVLICFRE
aa sub L A T
aaTlr4 .....A....G.....SL-.....R....T..D.S..ER....E....N.....Y.SD
ceTlr4 .....A.....SS-T.....T..A.S.RGRR...G....N.....L.G
faTlr4 .....A..Q.....Y-K...C.....L...D..ER....E..I.N.....SSG
hrTlr4 .....K.....A....G..NV.....SI-.....T.....D.S.R.R....E....N.....FY..D
lsTlr4 .....A..Q.....Y-...PC.....L...D..ER....G..I.....SSG
pdTlr4 .....L...A.....SS-.....G.S..GRR...E....D.....L.D
tgTLR4 .....A.....KP-T...A.....NIER....EGR..D.....L.G
ggTLR4 .....T.....Y.QS.S.AK...G..I...S...LNA.HS.....N.....TNILKITA.QN...S.....Q....M....
mmTLR4 QF...NPQV..S.DM...P.DF.QDQ..Q..K.H..T..GN.NSSNI.K.C..N....H.H...L...K.ER..EI.EPSIME...D.TID..R.TYTND
hsTLR4 RV.HQMPL...S.D....PMNF.Q....KE.R.HK.T..NN.DSLNV.K.CI.....E.H..VL....NEG..EK.DKSA.E...NLTI...R.AYLDY
300 310 320 330 340 350 360 370 380 390
LRR6
pmTlr4 FEDDTDTLFNCIGNVSTVRLVDLGLEEISQVPARSKVKQLECKKCSFEDVPALKLSLFKELRVLRITKNKRLKNFSQNFEGLTNLEVIDLSENRLTFSSC
aa sub Q
aaTlr4 ...Y.....V.....I...G..M.....VW.....K.K....S....M.....S....R.
ceTlr4 ...G..S.....I.....VG.....G.D.....RS....E.K....S.....
faTlr4 A.....N..H.A..A..I.....I...P....W.....Q.....Q.HV....E.E....SD.....R.
hrTlr4 .....V.....I...G.....W.Q.....E.....G....R.K....SH.....S....R.
lsTlr4 A.H..EN..H.A..A..I.....I...P....WP.....Q.....Q.HV....E.E....SD.....R.
pdTlr4 .....V.....I.....E..VW....H.....D.....R.....TS....K.K....S.....R.
tgTLR4 .K.....V.....I.....K.....VW...R.....H.....Q.....ND.RN..T.EHK.K..S.....
ggTLR4 .N.....D.....T.I.....N..TL.E..MF.Q..H..W.R.K.QEL..E.....S.D.NG.E.K.GS..Y...V.....S.LT.
mmTLR4 .S..IVK-.H.LA...AMS.AGVSIKYLED..KHF.WQS.SIIR.QLKQF.T.D.PFL.S.---TL.M..GSIS.KK--VA.PS.SYL...R.A.S..G.
hsTLR4 YL.DID....LT...SFS..SVTI.RVKDFSYNFGWQH..LVN.K.GQF.T...KSL.R.---TF.S..GGNA..E--VD.PS..FL...R.G.S.KG..

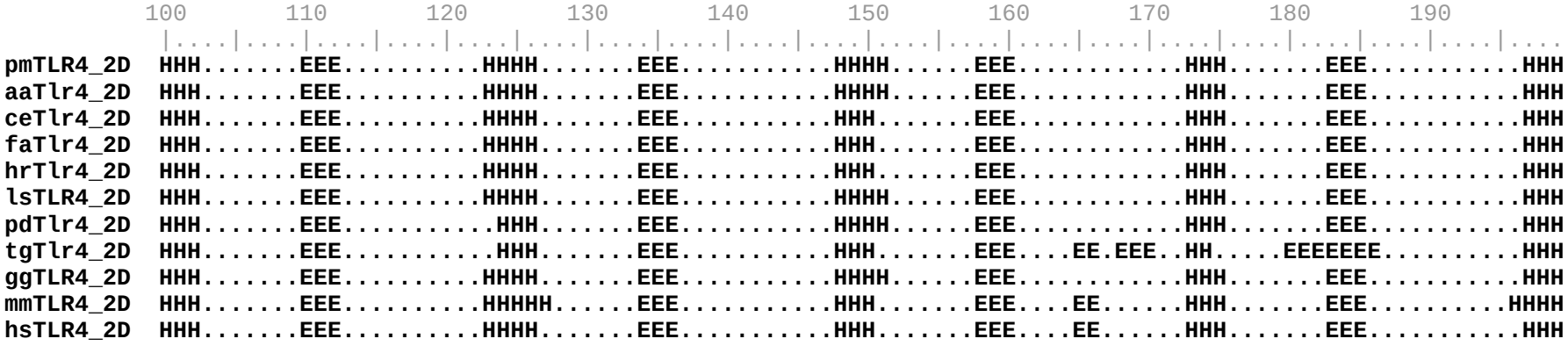

400 410 420 430 440 450 460 470 480 490
LRR7 LRR8 LRR9 LRR-CT
pmTlr4 CSPQFQNCPNLKHLNLSFNSNIRLTGDFTNVKNLLYLDLQHTTLFGPGSYPVFLSLQKLIYLDISHTKTEVKSQCTFCGLNSLQVLKMAGNSFEGNKLAG NFKNLSHLHTLDISSCKLEHVDQSTFDALSELKELNISNNKLLTFDPGVYQPLRALRVLDFSRNQLTVLLHSAWEILPDSLVLLDISQNLFDCSCVYLDF
aa sub A K I D R
aaTlr4 .....F.....R.....D....N .....S.....Q.....D..R.....L.....H...
ceTlr4 .....Y.....I..E.....R.....AD....N .....Q.....I...D..R.....L.....
faTlr4 .....L.....E.....R.....N....N .....S.A.....Q.....D..R.....
hrTlr4 .....R.....D....N .....S.....KS.....D..R.....H...
lsTlr4 .....L.....E.....R.....N....N .....I.....A.....S.A.....Q.....D..R.....
pdTlr4 .....V..N.....F.....D.....R.....A.....N .....Q.....N.....Q...I.....D.SRK.....
tgTLR4 .....R.....Y.....AD....N .....M....V.....Q.....A...DP.R.....
ggTLR4 ...K.PRS.....D.S...E.A.LR.....K.IHH.T.....L.....Y...H.M.HLI.H.....N.T.TN ..E.VRR.RI.....VW.....N.....I.....VT.K..Q..TA....N..MSF.SD..L.....H...E...TH.N.
mmTLR4 ..YSDLGTNS.R..D....GA.IMSAN.MGLEE.QH..F..S..KRVTEFSA....E..L.....Y.N.KIDFDGI.L..T..NT.....KD.T.SN V.A.TTN.TF..L.K.Q..QISWGV..T.HR.QL..M.H.N..FL.SSH.NQ.YS.ST..C.F.RIETSKG-ILQHF.K..AFFNLTN.SVA.I.EHQK.
hsTLR4 ..QSDFGTTS..Y.D....GV.TMSSN.LGLEQ.EH..F..SN.KQMSEFS.....RN.....H.R.AFNGI.N..S..E.....QE.F.PD I.TE.RN.TF..L.Q.Q..QLSPTA.NS..S.QV..M.H.NFFSL.TFP.KC.NS.Q...Y.L.HIMTSKKQELQHF.S..AF.NLT..D.A.T.EHQS.

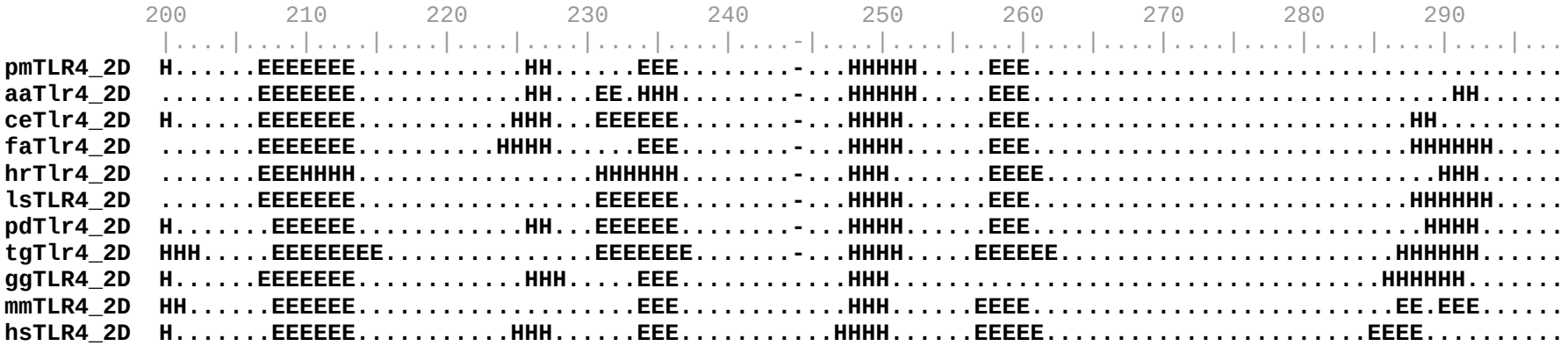

600 610 620 630 640 650 660 670 680 690
transmembrane domain
TIR
pmTlr4 LTWIKEKQELLQNKELMLCHTPSYTVNVSLPSFDLSSCHVSAGKVASSVVVLLCVVVFLFLIYKYYFQLYYSVVLLSGCKHSAERGDTYDAFVIHSSKDQ
aa sub V
aaTlr4 .E.V..E.....VA.....M.....I..L.....Y.....
ceTlr4 .E.....VA.....IS.....F...L...V.....Y.....
faTlr4 .E....R.....MA.....NTA.....L.L.....FM.....
hrTlr4 .E.V.G.....SE.....VA....A.....IS....A.....L.....Y.....
lsTlr4 .E....R.....VA.....L..A.....L.L.....
pdTlr4 .K.....R....VA....AR.....Y..V..IS.....L...V.....Y.....
tgTLR4 .K.V.....E.....VA.....I...Q...P....F.....V.R.....M.....Y.....
ggTLR4 .K.V....D..Q..HS.I....A.MK.M..SN..M....PNPTT..C..T...AAG.....L.....I.....
mmTLR4 .Q.V..QKQF.V.V.Q.T.A..VEMNTSLVLD.NN.T.YMYKTIISV..SVIV.STVA....HF..H.----I.IA...KYSRGESI.....Y..QNE
hsTLR4 .Q...DQRQ..VEV.R.E.A...DKQGMPVL.LN-IT.QMNKTIIGV..LSV.V.S.VAV.V..F..H.----M..A..IKYGRGENI.....Y..Q.E
700 710 720 730 740 750 760 770 780 790
pmTlr4 EWVTKELVEPLEGGAPPFRLCLYYRDFLPGVPVVTNIIQEGFLSSRNVIAVISADFLESKWCSFEFDIAQSWQLVEGKTGIIMIVLENVDKALLRQRLGL
aa sub I I N
aaTlr4 .....R...H.....I.....S.....A.....D.N.....
ceTlr4 .....R...H.....I.....A.....N.D.....
faTlr4 .....H.....I.....GD.....
hrTlr4 .....K...H.....I.....T.....D.N.....
lsTlr4 .....H.....I.....D.N.....
pdTlr4 .....R...H.....I.....T.....A.....D.....
tgTLR4 ...M.....T.R.....I.....E.....Q.....A.L.....D.N.....
ggTLR4 ...M.....E.K...Q....F.....I.....R.....A.....I.GE...T.....
mmTLR4 D..RN...KN..E.V.R.H...H....I...AIAA.....HK..K..V.V.RH.IQ.R..I..YE...T..FLSSRS...F....K.E.S....QVE.
hsTLR4 D..RN...KN..E.V...Q...H....I...AIAA...H...HK..K..V.V.QH.IQ.R..I..YE...T..FLSSRA...F...QK.E.T....QVE.

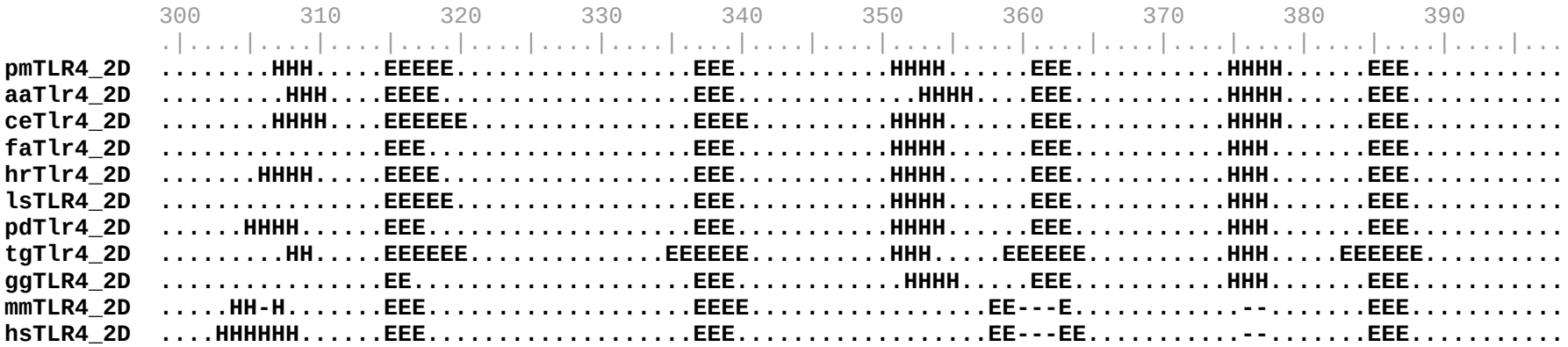


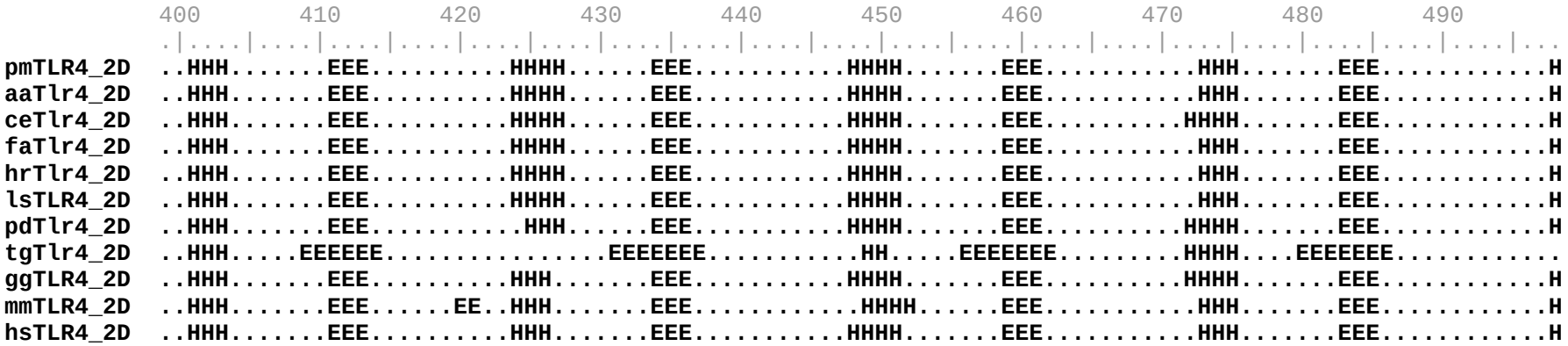
S4 Alignment of TLR4 amino acid sequences of all described passerines and the other selected model spesies (zebra finch, tgTLR4, ACN58232; chicken, ggTLR4, AAL49971.1; mus, mmTLR4, AAF05317; human, hsTLR4, AAF05316). Numeral axis is adjusted according to pmTLR4. Amino acid substitutions in pmTLR4 are marked in the separate row (**aa sub**), the crucial Q549R is moreover marked with arrow. Known polymorphic sites in other species are highlighted by grey and amino acid substitutions crucial in desease susceptibility are highlighted by black. The asterisks (*****) indicate MD2-binding site and crosses (#) indicate LPS-binding sites.


0 10 20 30 40 50 60 70 80 90
pmTLR4_2D -....HHHHHHHHHHHHHH.....HHHH.....EEE.....EEEEE.....HHH.....EEE.....
aaTlr4_2D -......HHHHHHHHHHHH.....HHH......EEEE.....EEEEE.....HHH.....EEE.....
ceTlr4_2D -........HHH...EEEEEE.....HHHH......EEE.....EEEEE.....HHH.....EEE.....
faTlr4_2D -......HHHHHHHHHHHH.....HHHH.....EEE.....EEEEE.....HHH.....EEE.....
hrTlr4_2D -......HHHHHHHHHHHH.....HHHH.....EEE.....EEEEE.....HHH.....EEE.....
lsTLR4_2D -......HHHHHHHHHHHH.....HHHH.....EEE.....EEEEE.....HHH.....EEE.....
pdTlr4_2D -.......HHHHHHHHHHH.....HHHHH.....EEE.....EEEEE.....HHH.....EEE.....
tgTlr4_2D -......HHHHHHHHHHHH.....HHH......EEE.....EEEEE.....HHH.....EEE.....
ggTLR4_2D -.......HHH..HHHHHHHH.....HHHH......EEE.....EEEEE.....HHH.....EEE.....
mmTLR4_2D ..H--HHHHHHHHHHH-HHH.....---...HHH.....EEE.....EEE.....EE..HHH.....EEE.....
hsTLR4_2D ...-...EEEHHHHHHHHHH.....---.....EEE.....EEE.....HHH.....EEE.....


100 110 120 130 140 150 160 170 180 190
pmTLR4_2D HHH.....EEE.....HHHH.....EEE.....HHHH.....EEE.....HHH.....EEE.....HHH
aaTlr4_2D HHH.....EEE.....HHHH.....EEE.....HHHH.....EEE.....HHH.....EEE.....HHH
ceTlr4_2D HHH.....EEE.....HHHH.....EEE.....HHH.....EEE.....HHH.....EEE.....HHH
faTlr4_2D HHH.....EEE.....HHHH.....EEE.....HHH.....EEE.....HHH.....EEE.....HHH
hrTlr4_2D HHH.....EEE.....HHHH.....EEE.....HHH.....EEE.....HHH.....EEE.....HHH
lsTLR4_2D HHH.....EEE.....HHHH.....EEE.....HHHH.....EEE.....HHH.....EEE.....HHH
pdTlr4_2D HHH.....EEE.....HHH.....EEE.....HHHH.....EEE.....HHH.....EEE.....HHH
tgTlr4_2D HHH.....EEE.....HHH.....EEE.....HHH.....EEE....EE.EEE..HH.....EEEEEEE.....HHH
ggTLR4_2D HHH.....EEE.....HHHH.....EEE.....HHHH.....EEE.....HHH.....EEE.....HHH
mmTLR4_2D HHH.....EEE.....HHHHH.....EEE.....HHH.....EEE....EE.....HHH.....EEE.....HHHH
hsTLR4_2D HHH.....EEE.....HHHH.....EEE.....HHH.....EEE....EE.....HHH.....EEE.....HHH


200 210 220 230 240 250 260 270 280 290
pmTLR4_2D
aaTlr4_2D
ceTlr4_2D
faTlr4_2D
hrTlr4_2D
lsTLR4_2D
pdTlr4_2D
tgTlr4_2D
ggTLR4_2D
mmTLR4_2D
hsTLR4_2D


300 310 320 330 340 350 360 370 380 390
pmTLR4_2D
aaTlr4_2D
ceTlr4_2D
faTlr4_2D
hrTlr4_2D
lsTLR4_2D
pdTlr4_2D
tgTlr4_2D
ggTLR4_2D
mmTLR4_2D
hsTLR4_2D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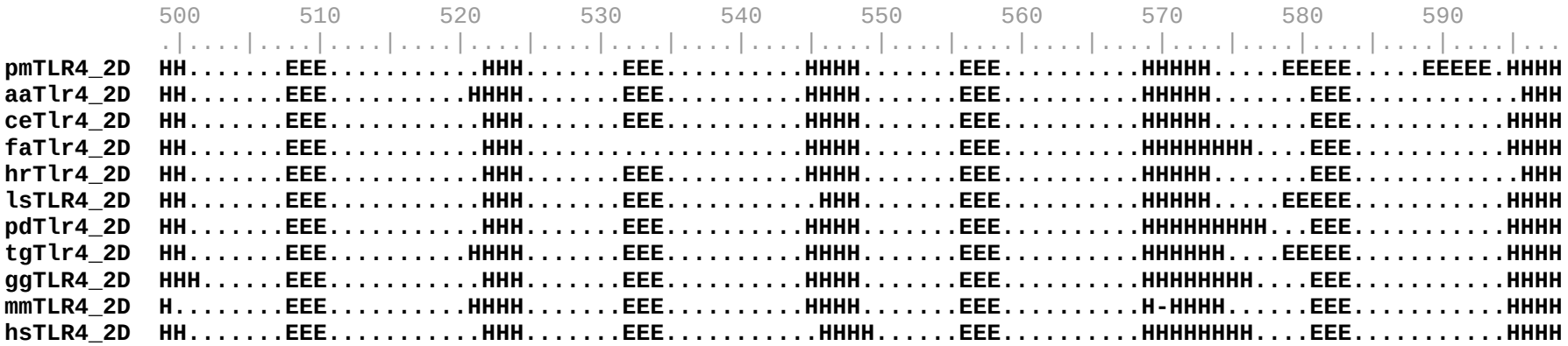
pmTLR4_2D ..HHH.....EEE.....HHHH.....EEE.....HHHH.....EEE.....HHH.....EEE.....H
aaTlr4_2D ..HHH.....EEE.....HHHH.....EEE.....HHHH.....EEE.....HHH.....EEE.....H
ceTlr4_2D ..HHH.....EEE.....HHHH.....EEE.....HHHH.....EEE.....HHHH.....EEE.....H
faTlr4_2D ..HHH.....EEE.....HHHH.....EEE.....HHHH.....EEE.....HHH.....EEE.....H
hrTlr4_2D ..HHH.....EEE.....HHHH.....EEE.....HHHH.....EEE.....HHH.....EEE.....H
lsTLR4_2D ..HHH.....EEE.....HHHH.....EEE.....HHHH.....EEE.....HHH.....EEE.....H
pdTlr4_2D ..HHH.....EEE.....HHH.....EEE.....HHHH.....EEE.....HHHH.....EEE.....H
tgTlr4_2D ..HHH.....EEEEEE.....EEEEEEE.....HH.....EEEEEEE.....HHHH....EEEEEEE.....
ggTLR4_2D ..HHH.....EEE.....HHH.....EEE.....HHHH.....EEE.....HHHH.....EEE.....H
mmTLR4_2D ..HHH.....EEE.....EE..HHH.....EEE.....HHHH.....EEE.....HHH.....EEE.....H
hsTLR4_2D ..HHH.....EEE.....HHH.....EEE.....HHHH.....EEE.....HHH.....EEE.....H


500 510 520 530 540 550 560 570 580 590
pmTLR4_2D HH.....EEE.....HHH.....EEE.....HHHH.....EEE.....HHHHH.....EEEEE.....EEEEE.HHHH
aaTlr4_2D HH.....EEE.....HHHH.....EEE.....HHHH.....EEE.....HHHHH.....EEE.....HHH
ceTlr4_2D HH.....EEE.....HHH.....EEE.....HHHH.....EEE.....HHHHH.....EEE.....HHHH
faTlr4_2D HH.....EEE.....HHH.....HHHH.....EEE.....HHHHHHHH....EEE.....HHHH
hrTlr4_2D HH.....EEE.....HHH.....EEE.....HHHH.....EEE.....HHHHH.....EEE.....HHH
lsTLR4_2D HH.....EEE.....HHH.....EEE.....HHH.....EEE.....HHHHH.....EEEEE.....HHHH
pdTlr4_2D HH.....EEE.....HHH.....EEE.....HHHH.....EEE.....HHHHHHHHH...EEE.....HHHH
tgTlr4_2D HH.....EEE.....HHHH.....EEE.....HHHH.....EEE.....HHHHHH....EEEEE.....HHHH
ggTLR4_2D HHH.....EEE.....HHH.....EEE.....HHHH.....EEE.....HHHHHHHH....EEE.....HHHH
mmTLR4_2D H.....EEE.....HHHH.....EEE.....HHHH.....EEE.....H-HHHH.....EEE.....HHHH
hsTLR4_2D HH.....EEE.....HHH.....EEE.....HHHH.....EEE.....HHHHHHHH....EEE.....HHHH

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           600       610       620       630       640       650       660       670       680       690
           .|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
pmTLR4_2D  HHHHHHHHHH.....EEEEE..HHH...EE.........HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHEEEEEEEEEE.............EEEE..HHHH
aaTlr4_2D  HHHHHHHHHH............HHH.........HH...HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH.....HHHH.....EEEE..HHHH
ceTlr4_2D  HHHHHHHHHH......EEE...HHH..............HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH.....EEEE..HHHH
faTlr4_2D  HHHHHH..........EEEE..HHH..............HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH..........EEEE..HHHH
hrTlr4_2D  HHHHHH..........EEE...HHH...EE.EEE.......HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH...........EEEE....HH
lsTLR4_2D  HHHHHH..........EEE...HHH..............HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH..........EEEE....HH
pdTlr4_2D  HHHHH...........EEE.........EEEE..HHH....HHHHHHHHHHHHHHHHHHHH..EEEEEHHHHHHHHHHHH.......EEEE....HH
tgTlr4_2D  HHHHHH..........EEEE..HHH..............HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH.....EEEEE...HH
ggTLR4_2D  HHHHHHHHHH......EEE..HHHH.........HHH...HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH.............EEEE..HHHH
mmTLR4_2D  HHHHHH..........EEE...HHH...EEEE..HHH....HHHHHHHHHHHHHHHHHHHHHHHHHH----HHHHH............EEEEE....H
hsTLR4_2D  HHHHHH..........EEE....HH...EE....-HH....HHHHHHHHHHHHHHHHHHHHHHHHHH----HHHHHHHH.......EEEEEEE...HH

           700       710       720       730       740       750       760       770       780       790
           .|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
pmTLR4_2D  HHHHHHHHHHHH......EEEEE........HHHHHHHHHHHHH..EEEEEE.HHHHH.HHHHHHHHHHHHHHHHH...EEEEEE.....HHH..HHHHH
aaTlr4_2D  HHHHHHHHHHHH......EEEEEE.......HHHHHHHHHHHHH...EEEEE.HHHHH.HHHHHHHHHHHHHHHHH...EEEEEE.....HHHH.HHHHH
ceTlr4_2D  HHHHHHHHHHHH......EEEEEE......HHHHHHHHHHHHH..EEEEEE.HHHHH.HHHHHHHHHHHHHHHHH...EEEEEE.....HHH..HHHHH
faTlr4_2D  HHHHHHHHHHHH......EEEEEE.......HHHHHHHHHHHHH..EEEEEE.HHHHH.HHHHHHHHHHHHHHHHH...EEEEEE.....HHH..HHHHH
hrTlr4_2D  HHHHHHHHHHHH......EEEEE........HHHHHHHHHHHHH..EEEEEE.HHHHH.HHHHHHHHHHHHHHHHH...EEEEEE.....HHHH.HHHHH
lsTLR4_2D  HHHHHHHHHHHH......EEEEE........HHHHHHHHHHHHH..EEEEEE.HHHHH.HHHHHHHHHHHHHHHHH...EEEEEE.....HHHH.HHHHH
pdTlr4_2D  HHHHHHHHHHHH......EEEEE........HHHHHHHHHHHHH..EEEEEE.HHHHH.HHHHHHHHHHHHHHHHH...EEEEEE.....HHH..HHHHH
tgTlr4_2D  HHHHHHHHHHHH......EEEEE........HHHHHHHHHHHHH...EEEEE.HHHH..HHHHHHHHHHHHHHHHH...EEEEEE.....HHH..HHHHH
ggTLR4_2D  HHHHHHHHHHHH......EEEEE........HHHHHHHHHHHHH..EEEEEE.HHHHH.HHHHHHHHHHHHHHHHH...EEEEEE.....HHH..HHHHH
mmTLR4_2D  HHHHHHHHHHHH......EEEEEE.......HHHHHHHHHHHHH...EEEEE.HHHHH.HHHHHHHHHHHHHHHHH...EEEEEE.....HHH..HHHHH
hsTLR4_2D  HHHHHHHHHHHH......EEEEEE.......HHHHHHHHHHHHH...EEEEE.HHHHH.HHHHHHHHHHHHHHHHH...EEEEEE.....HHH..HHHHH
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          800       810       820       830       840       850
          .|....|....|....|....|....|....|....|....|....|....|..
pmTLR4_2D HHHH....EEE........HHHHHHHHHHH.............
aaTlr4_2D HHHH....EEE........HHHHHHHHHHH.......HH..EE.
ceTlr4_2D HHHH....EEE........HHHHHHHHHHH...........EE.
faTlr4_2D HHHH....EEE........HHHHHHHHHHH.......HHHH...
hrTlr4_2D HHHH....EEE........HHHHHHHHHHH...........EEE.
lsTLR4_2D HHHH....EEE........HHHHHHHHHHH.......HH.EEE.
pdTlr4_2D HHHH....EEE........HHHHHHHHHHH...........EE.
tgTlr4_2D HHHH....EEE........HHHHHHHHHHHH........HHHH.
ggTLR4_2D HHHH....EEE........HHHHHHHHHH............EE.
mmTLR4_2D HHHH....EEE........HHHHHHHHHHH..............--.HH.....
hsTLR4_2D HHHH....EEE........HHHHHHHHHH.......................
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S5 Alignment of TLR4 secondary structures of all described passerines and the other selected model species (zebra finch, tgTLR4, ACN58232; chicken, ggTLR4, AAL49971.1; mus, mmTLR4, AAF05317; human, hsTLR4, AAF05316). Numeral axis is adjusted according to pmTLR4. Alpha-helixes (H) and beta-sheets (E) are present. The amino acids that are not include into any structural unit are mark as points (.).